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Process for partitioning of molecules

Field of the invention

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The present invention relates to isolation and purification of proteins in aqueous two-phase systems (ATPS). Specifically the invention provides processes for partitioning of molecules of interest in ATPS by fusing said molecules to targeting proteins which have the ability of carrying said molecule into one of the phases.

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Background of the invention

Liquid-liquid extraction in an aqueous two-phase system (ATPS) can offer a powerful technique for isolation and purification of proteins. The separation of macromolecules and particles by means of liquid-liquid extraction is well known (Albertsson, 1986; 15 Walter *et al.*, 1985; Kula, 1990). Mainly polyethylene glycol (PEG) - salt, PEG-dextran and PEG-starch systems have been in use. More recently detergents and detergents with reversed solubility were discovered as suitable methods for separation of macromolecules, and especially for the separation of proteins.

20

An advantage of aqueous two-phase systems (ATPS) is that they are especially suited for large scale processing of microbial proteins not only from culture supernatants but also from crude extracts containing cells and cell debris (Kula, 1979; Kula, 1985). Characteristic features of biological fluids as well as suspensions are rather small 25 particle sizes, low density differences between fluid and suspended solids, high viscosities of the extracts and high compressibility of the solids (Hustedt *et al.*, 1985; Bender and Koglin, 1986). These attributes decrease the performance of conventional methods for solid-liquid separation like centrifugation and filtration at the beginning of a protein recovery process. Using an aqueous two-phase system removal of solids can be integrated into a liquid-liquid separation step, clarification is thus combined with an 30 initial purification (Kula, 1979; Kula, 1985).

After the extraction process phase separation can be accomplished by settling under gravity as well as by centrifugation (Kula, 1985). ATPS can be applied in various scales

from very small laboratory scale to large industrial scale thus suiting for various proteins, purposes and needs. With regard to industrial purposes commercially available centrifugal separators can be used to shorten separation time. Several authors have investigated the potential of centrifugal separators of various design for processing of large volumes of aqueous two phase systems (Kula, 1979; Kula *et al.*, 1981, Kula *et al.*, 1982; Kula, 1985). In these studies the authors have used polymer/polymer or polymer/salt systems and the results of these investigations demonstrate the feasibility of continuous separation of aqueous two-phase systems in centrifugal separators.

Extraction systems based on nonionic surfactants have been described as an alternative to standard polymer/polymer or polymer/salt systems. Phase forming surfactants are e.g. polyoxyethylene type nonionic detergents. The basis of this type of aqueous two-phase system is the temperature-dependent reversible hydration of the polar ethylene oxide head groups. The temperature at which the phase separation occurs is referred to as the cloud-point (cloud-point extraction). This kind of aqueous two-phase system is especially suited for the extraction of amphiphilic biomolecules. The potential of this type of two-phase system for separating membrane bound proteins from cytosolic and peripheral membrane proteins was first demonstrated by Bordier (1981). Heusch and Kopp (1988) have been able to demonstrate that lamellar structures formed in the miscibility gaps of polyglycol ether / water systems are responsible for the selective extraction of hydrophobic substances.

Recently, the successful application of a surfactant-based aqueous two-phase system for the extraction of a membrane bound protein (cholesterol oxidase) from the unclarified culture medium of the gram-positive microorganism *Nocardia rhodochrous* on a bench scale has been reported (Minuth *et al.*, 1995). By addition of only one chemical compound a product release through solubilization was possible in homogeneous phase and in a second step a clarification as well as an initial purification was achieved by an extraction process at elevated temperatures separating the detergent rich phase. A closed concept was further developed for the production of the membrane bound enzyme by surfactant-based extraction, organic solvent extraction and anion-exchange chromatography, which gave a product suitable for analytical applications (Minuth *et al.*, 1996).

In aqueous two phase systems the desired target e.g. a protein should partition selectively into one phase (preferentially the lighter phase) while the other substances should partition into the other phase (preferentially the heavier phase). In PEG/salt and PEG/dextran and similar systems there are several driving forces for a substance like charges, hydrophobic, hydrophilic forces or the dependence on conformation or ligand interaction (Albertsson, 1986). The forces leading to separation in detergent based aqueous two phase systems are suggested to be primarily hydrophobic (Terstappen *et al.*, 1993). Even if a lot of work has been carried out in the field of prediction in ATPS, none of the designed models provides a physical picture of the phase behaviour and prediction is hardly possible (Johansson *et al.*, 1998).

In ATPS the partitioning coefficient is defined as the concentration (activity in case of an enzyme) of the target in the top phase divided by the concentration (enzyme:activity) of the target protein in the bottom phase.

$$K = \frac{c_{i,T}}{c_{i,B}}$$

Yield: is defined as the amount of target in the top phase divided by the sum of the amount of target in top and bottom. This leads to the following equation

$$Y_T = \frac{1}{1 + \left[\frac{V_B}{V_T} \cdot \frac{1}{K} \right]}$$

If the desired substance is directed to the heavier phase (as it can be the case using Triton) the yield is defined by

$$Y_B = \frac{1}{1 + \left[\frac{V_T}{V_B} \cdot K \right]}$$

The volume ratio of the two coexisting phases are defined by the volumes of the lighter over the heavier phase, respectively.

$$R = \frac{V_T}{V_B}$$

An example of useful proteins facing problems in purification in a cost-effective way are the commonly used industrial enzymes used as biocatalysts, the glycosyl hydrolases, proteases and lipases produced by fungi and bacteria. These are used in e.g. laundry, textile, paper and pulp, food and feed industry. The fact that microbes produce many different enzymes during their growth and the fact that some of these may be undesired in certain applications leads to a need to enrich the active component(s). This enrichment can be performed by choosing appropriate growth conditions, by genetic engineering and/or by down-stream processing (e.g. purification of the active component(s)).

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Purification of proteins are generally performed by chromatography. Usually gel-chromatographic methods are used based on ion-exchange, hydrophobic interaction, affinity chromatography and molecular sieving. Methods like electrophoresis and crystallisation can also be used. These methods are well known in the art and suitable for proteins of fairly high market value. In case of bulky enzyme production these methods, however, are too expensive in order to keep the final product on a compatible price level. Due to similar properties of these enzymes several purification steps are usually needed to separate the proteins from each other. This often causes low final yields and therefore a high loss of product.

20

Many extracellular hydrolases produced by the filamentous fungus *Trichoderma* are currently used in different industrial applications in large scale. These hydrolases are e.g. hemicellulases (such as xylanases and mannanases), cellulases (such as endoglucanases and cellobiohydrolases) and proteases. Purification of these is well known in the art (Bhikhabhai *et al.*, 1984; Pere *et al.*, 1995), but for large industrial applications the purification methods are too expensive. Alternative methods to enrich these hydrolases have been used, including deletion of undesired genes by genetic engineering (Suominen *et al.*, 1992). However, even after extensive genetic engineering some minor undesired activities may still be present in the final product.

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ATPS have been studied in purification of cellulases of *T. reesei* and the purification of an endoglucanase III showed some promising results, enriching the yield of the protein in the upper phase (U.S. Pat. 5,139,943). ATPS have also been studied in purification

of lipases, endoxylanase and natamycin (EP 0 574 050 A1). No K and Y values are, however, mentioned.

As in other protein purification methods, similar properties of proteins produced by an organism impair also in ATPS, e.g. selective separation of one protein is not achieved optimally. To obtain selectivity in purification affinity chromatographic methods are used especially for analytic purposes and in purification of high-value products. These include immunoaffinity chromatography and various fusion protein strategies well described in the art such as fusing the protein of interest to an other protein (e.g. glutathione-S-transferase), protein domain (e.g. protein A-ZZ domain) or small peptide (e.g. His-tag), which selectively bind to the solid carrier and thus the recovery of the fusion partner is obtained as well. The fusion protein can be suitable for the particular purpose as such or cleavage of the product from the added fusion partner may be desired. There are well-known methods in the art on cleavage of fusion proteins from their partners by proteases, e.g. by factor X, thrombin or papain or by genetically introducing a protease cleavage site (e.g. Kex2 site) or autoprocessing domains (e.g. Intein, New England Biolabs) or by chemical cleavage.

ATPS offer advantages mainly with respect to technology compared with the solid state based separation systems e.g. affinity column-based techniques. The scale-up of extractive enzymes is relatively simple utilising commercially available equipment and machinery common in the chemical industry. In addition, it can be used in a continuous process and it can be relatively cost extensive. It can be used as a single step for clarification, concentration and purification. ATPS can be used as a first capture-step, but for bulk products often no further purification is needed.

To aid selective separation in two-phase systems, recent publications have described the fusion of small peptide tags of 12 amino acids to the protein to be purified. The most successful of these soluble peptides are containing tryptophans. So far they have mainly been applied for very small molecules like the staphylococcal protein A derivative ZZT0 (Berggren *et al.*, 1999; Hassinen *et al.*, 1994; Köhler *et al.*, 1991).

Use of ATPS has so far been limited to certain targets. Due to the advantages of ATPS in protein separation, purification and localisation, highly selective and powerful methods should be developed. This is especially important for large scale processes where ATPS in general is very inexpensive as a first capture step or as the only step for purification, clarification and concentration. The system should be universal so that the technique would be strong enough to mediate separation of in principle any component to the desired phase irrespective of its size or biochemical properties.

Description of the invention

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In this invention we describe selective separation and partitioning of molecules and particles fusing them with targeting proteins having the capability to carry the molecule or particle of interest to the desired phase in ATPS, and to keep it in this phase if wanted. This invention is directed to make ATPS usable for every biotechnological product. By addition of the targeting protein to selective products, either by genetic tagging of proteins, by chemical binding, glueing or by use of any other technique, the product molecule can be turned more suitable for separation in ATPS. Using ATPS the product or certain component is therefore driven to one phase while the other components or by-products are directed to the other phase(s).

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We also describe that efficient separation in ATPS can be obtained using targeting proteins which are/can be larger than the described small soluble synthetic peptide tags of 12 amino acids or less. These targeting molecules can aid in separating of small molecules but even large proteins and particles. Unlike the small peptide tags, it is not necessary that they contain tryptophan residues, although they may do so. They can be hydrophobic or moderately hydrophobic and/or amphipathic in nature, either in monomeric form or when forming aggregates. Such proteins can be found in nature or they can be designed, or obtained through for instance methods known in art for mutant generation, gene shuffling or directed evolution. Suitable targeting molecules can be screened for instance by fusion the product of interest to a library of natural or mutant sequences, and screening the ability of the fusion molecules to separate in ATPS. Furthermore, any molecule capable of separating in ATPS is a suitable targeting molecule.

30

Several techniques exploiting purified protein for isolation of the corresponding gene may be used to find genes encoding suitable targeting molecules for ATPS. Suitable proteins or polypeptides may be purified on the basis of their properties. They can be obtained by applying the cells, cell extracts or culture media to ATPS and recovering the proteins or peptides separated into the phase containing the heavier hydrophobic phase material. Suitable targeting molecules may also be recovered for example from the culture medium foam formed either during the cultivation of a microorganism or caused by bubbling gas through the medium. Proteins and peptides suitable as targeting molecules may further be recovered from aggregates caused by freezing of culture media. After the targeting molecules have been purified, the corresponding genes are isolated using techniques known to a person skilled in the art. Such techniques include for example screening of expression libraries using antibodies raised against purified polypeptide or peptide, and PCR cloning and screening of genomic and/or cDNA libraries with oligonucleotides designed on the basis of N-terminal or internal protein sequences.

Examples of molecules suited as targeting proteins in ATPS found in nature are hydrophobin-like small proteins. Hydrophobins are secreted proteins with interesting physico-chemical properties that have recently been discovered from filamentous fungi (Wessels, 1994; Wösten and Wessels, 1997; Kershaw and Talbot, 1998). One characteristic feature of these proteins is their moderate hydrophobicity. They are usually small proteins, approximately 70 to 160 amino acids, containing eight cysteine residues in conserved pattern, and do usually not contain tryptophanes. However, also multimodular proteins with one or several hydrophobin domains and e.g. proline-rich or asparagine/glycine repeats, or hydrophobins containing less than eight cysteine residues have been characterized (Lora *et al.*, 1994; Lora *et al.*, 1995; Arntz and Tudzynski, 1997). Hydrophobins have been divided into two classes based on their hydropathy profiles (Wessels, 1994).

Today most protein data exists for the hydrophobins Sc3p of *Schizophyllum commune* (class I), and cerato-ulmin of *Ophiostoma ulmi* and cryparin of *Cryphonectria parasitica* (class II), although more than 30 gene sequences for hydrophobins have been published (Wösten and Wessels, 1997). HFB genes are often naturally highly expressed but due

to special requirements in cultivation conditions and the biochemical properties of the proteins, purification of HFBs in large amounts have been difficult. For instance only relatively moderate production levels of a few mg per liter of Sc3 hydrophobin in static cultures are obtained (Han Wösten, personal communication). Published purification procedures include e.g. multi-step extraction from fungal cell walls using organic solvents and bubbling or freezing of culture filtrates (Wessels, 1994). No reports of successful production of hydrophobins are available; levels of cerato-ulmin were no higher than those obtained with other naturally occurring fungal isolates (Temple *et al.*, 1997).

Upon shaking hydrophobin-containing solutions, the protein monomers form rodlet-like aggregates. These structures are similar to the ones found on surfaces of aerial structures. The self-assembly of purified Sc3 hydrophobin into a 10 nm thick amphipathic layer on hydrophilic and hydrophobic surfaces has been demonstrated (Wösten *et al.*, 1994a; Wösten *et al.*, 1994b). This film is very strongly attached to the surface and not broken, for instance, by hot detergent. The hydrophobic side of the layer on hydrophilic surfaces shows properties similar to teflon (Wessels, 1994). The Sc3 assemblages, as well as those of cerato-ulmin and cryparin, also form on gas-liquid or gas-air interphases thus stabilizing air bubbles or oil droplets in water.

Surface activity of proteins is generally low but hydrophobins belong to surface-active molecules, their surfactant capacity being at least similar to traditional biosurfactants such as glycolipids, lipopeptides/lipoproteins, phospholipids, neutral lipids and fatty acids (Wösten and Wessels, 1997). In fact Sc3 hydrophobin is the most potent biosurfactant known. It lowers the water surface tension to 24 mJm^{-2} at a concentration of $50 \mu\text{g/ml}$ due to a conformational change during self-assembly of monomers into an amphipathic film (Wösten and Wessels, 1997).

Hydrophobin-like molecules vary in their properties. For instance, rodlet-forming capacity has not been assigned for all hydrophobins (such as some class II), or they might have a weaker tendency to form stable aggregates (Russo *et al.*, 1992; Carpenter *et al.*, 1992). Another group of fungal amphipathic proteins are repellents (Wösten *et al.*, 1996 (Ustilago), for review, see Kershaw and Talbot, 1998). Consequently, other type

of proteins suited as targeting proteins for ATPS, may have only some of the features assigned to hydrophobins. Other suitable proteins are hydrophobic ones such as e.g. lipases, cholesterol oxidase, membrane proteins, small peptide drugs, aggregating cell wall proteins, lipopeptides or any parts of these or combinations of these, and other molecules like glycolipids, phospholipids, neutral lipids, fatty acids in combination with proteins or peptides.

In this invention the targeting protein, such as a hydrophobin-like protein or parts of it, is bound to the product molecule or the component to be separated. First, phase forming materials and eventually possibly also additional salts are added to a watery solution containing the fusion molecule or component, and optionally also the contaminating materials. The added agents are mixed to facilitate their solubilization. As soon as they are solubilized the two phases are formed either by gravity settling or centrifugation. In the separation the target protein drives the product to for instance the detergent-rich phase which could either be the top or the bottom phase. The method is not only useful for purification of products of interest but also for keeping the product or the component of interest, such as a biocatalyst, in a particular phase which enables certain useful biotechnical reactions.

Several ATPS systems are suitable for performing this invention. These include PEG containing systems, detergent based systems and novel thermoseparating polymers. Detergent based systems can be nonionic, zwitterionic, anionic or cationic. The system can be based on amphiphilic polymeric detergents, micelle forming polymers. Novel polymers can be based on polyethylene-polypropylene copolymers such as pluronic block copolymers, Brij, polyoxyethylene derivatives of partial ethers of fatty acids made by adding polyoxyethylene chains to the nonesterified body and polyoxyethylene derivatives. The well known PEG/salt, PEG/dextran and PEG/starch (or derivatives such as Reppal, hydroxipropyl starch) systems where PEG and water are forming the top phase and dextran/starch/salt and water are forming the bottom phase. As salts are used phosphate, citrate, sulfate or others. In the present process the target is partitioning mainly to the top phase, while most of the contaminants are separating mainly to the bottom phase. Some hydrophobic contaminants might partition to the top phase as well. Using detergent based systems only one phase forming detergent has to be added.

Optionally, salts and other chemicals can be used in addition. The mentioned chemicals are added, and the solution is mixed. After mixing the separation can take place either by centrifugation or gravity settling. In order to separate into two phases the temperature of the solution has to be over the cloud-point of the detergent. The solution has to be
5 heated if the cloud-point is not reached otherwise. If wanted, a second separation step can follow after a first extraction step and the product rich phase can be further purified. Also the remaining product in the product poor and by-product rich phase can be re-extracted. Very good K values can be obtained and the yields and concentration factors are high.

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The process of the present invention can advantageously be used in laboratory scale but is especially suitable for large scale separations. It can successfully be used in the separation of proteins and components from large fermentations. Using genetic modifications, the method can be used to purify any protein of interest including
15 extracellular enzymes and proteins such as cellulases and hemicellulases from mixtures containing large amounts of protein such as several grams per liter. Furthermore, this separation can be obtained from various culture media including industrial media containing particular materials such as cellulose and spent grain. The method can be used to purify the product from culture media of strains modified not to produce
20 endogenous hydrophobins. The separation can be done directly from the fermentation broth which can additionally contain cells, even viscous filamentous fungi. High biomass levels are acceptable for the process as explained in example 9. An example is the extracellular endoglucanase I from the fungus *Trichoderma reesei* which can be tagged for instance with the class 2 HFBI and can for example be separated with the nonionic polyoxyethylene C12-C18EO5. In this example the detergent rich phase is the lighter
25 phase and contains most of the tagged endoglucanase while most of the other cellulases, proteases and other enzymes remain in the heavier phase. The mycelium separates to the bottom phase, too. The separation can be achieved using separation temperatures higher than 25°C. The temperature can be decreased if certain salts like NaCl or K₂SO₄ are
30 added.

The invention describes separation of molecules produced in various different organisms such as bacteria, yeast and filamentous fungi. The invention is suitable for purification

of product molecules from extra- or intracellular locations, including cell wall bound molecules. It provides examples how the fusion molecule can be secreted by these different organisms but also provide an example how the fusion can be produced intracellularly.

5

The invention further describes how fusion molecules consisting of several domains can be constructed and successfully expressed and produced. The invention describes fusions of the targeting molecule to a small protein (CBD), to a moderately sized protein (EGI) and to a huge highly glycosylated protein (FloI), and different domain variations of these. These molecules can be ready as such for biotechnical use. Alternatively, the product can be cleaved from the targeting protein by any method known in the art such as with proteases e.g. thrombin, factor X, papain or by chemical cleavage. Furthermore, ATPS is a preferential means to be used to separate the product from the targeting protein after cleavage, or these can be separated with other methods known in the art.

15

A suprising feature is that the targeting protein can also be used to carry large particles to the desired phase in ATPS. This can be obtained if the particles already contain proteins suited for targeting such as spores/conidia do in case of fungi. The targeting protein can also be attached to the particles or compounds *in vitro*. If cells are separated, the targeting protein can alternatively be expressed in the recombinant cells in such a way that it is exposed at the cell surface whereby it mediates the separation of the cells in ATPS. A teaching how this can be done is provided in example 22. Other types of molecules which direct the targeting molecule to the cell surface can be found e.g. in the literature including bacterial outer membrane proteins and lipoproteins (Ståhl and Uhlén, 1997), and yeast proteins α -agglutinin and flocculin (Schreuder *et al.*, 1996; Klis *et al.* (1994) WO 94/01567; Frenken (1994) WO 94/18330).

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A further advantage of the system is that the invention combined with ATPS provides a means to separate the product or desired component not only from other unnecessary or unwanted proteins but also from harmful proteins such as proteases as described in example 6. Thus, the invention is particularly suited for production and purification of heterologous proteins, e.g. sensitive mammalian proteins usually produced in limited amounts in heterologous hosts. Such proteins are for instance antibodies or fragments

thereof, interferon, interleukin, oxidative enzymes and any foreign protein which can otherwise be produced in a host. It is possible that separation of the product from e.g. culture medium can also be obtained on-line or semi-continuously, thus minimising the effect of proteases or other harmful components present in the culture. When produced
5 intracellularly, the invention also provides means to separate the heterologous product, for instance the inclusion bodies it may form, from the cellular extracts.

This invention describes for the first time that fusion proteins containing hydrophobin-like molecules can be made and produced in significant amounts despite the very
10 particular properties of hydrophobin-like molecules. Importantly, this invention also describes how recombinant strains producing increased amounts of hydrophobin-like proteins as such can be made. This provides means to produce the targeting protein for uses in which it is wanted that the targeting protein is bound to the product or particle *in vitro*, to enable further separation of such molecules or particles in ATPS.

15 Importantly, this invention also describes how hydrophobin-like molecules can be purified in ATPS very efficiently. The molecules can be separated in the same way as the above mentioned fusions, for instance by PEG systems or by detergent-based systems. Separation can be done from the culture medium or from cells. This provides
20 a significant improvement in making pure preparates containing hydrophobin-like molecules since due to their properties their purification is very complicated and results in losses with the previously reported techniques as described above.

The invention is further illustrated by the following Examples which describe
25 construction of the fusion molecules of the invention, and partitioning of the molecules of interest using the process according to the invention.

EXAMPLES

Example 1

Construction of vectors for expression of EGI and EGIcore HFBI fusion proteins
 5 under the *cbh1* and *gpd1* promoters of *Trichoderma* and *gpdA* promoter of
Aspergillus

For construction of an EGI-HFBI fusion protein, *hfb1* (SEQ ID 1) coding region (from
 Ser-23 to the STOP codon) and a peptide linker (Val Pro Arg Gly Ser Ser Ser Gly Thr
 10 Ala Pro Gly Gly) preceding it was amplified with PCR using pTNS9 as a template and
 as a 5' primer TCG GG **CACTACGTG** C CAG TAT AGC AAC GAC TAC TAC
 TCG CAA TGC *CTT GTT CCG CGT GGC TCT* AGT TCT GGA ACC GCA (SEQ
 ID 2) and as a 3' primer TCG TAC **GGA TCC** TCA AGC ACC GAC GGC GGT (SEQ
 ID 3). pTNS9 has been described in detail in Example 19. The sequence in bold in the
 15 5' primer encodes 16 C-terminal residues of EGI. The sequence in italics is a thrombin
 cleavage site and the underlined CACTACGTG is a DraIII site. The underlined
 GGATCC in the 3' primer is a BamHI site. The 280 bp PCR fragment was purified
 from agarose gel and ligated to pGEM-T T/A vector (Promega) resulting in pMQ102.

20 For construction of an EGIcore-HFBI fusion protein, the *hfb1* coding region (as above)
 was amplified with PCR using pTNS9 as a template and as a 5' primer ACT ACA
 CGG AG **GAGCTC** G ACG ACT TCG AGC AGC CCG AGC TGC ACG CAG
 AGC AAC GGC AAC GGC (SEQ ID 4) and as a 3' primer SEQ ID 3. The sequence
 in bold in the 5' primer encodes amino acids 410-425 in EGI and the underlined
 25 GAGCTC is a SacI site. The 260 bp PCR fragment was purified from agarose gel and
 ligated to pPCRII T/A vector (Invitrogen) resulting in pMQ111.

In the next step *Trichoderma* expression vectors for production of EGI-HFBI and
 EGIcore-HFBI fusion proteins under the control of *cbh1* promoter and terminator
 30 sequences were constructed. The expression vector used as a backbone in the constructs
 is pPLE3 (Nakari *et al.* (1994) WO 94/04673) which contains a pUC18 backbone, and
 carries the *cbh1* promoter (SEQ ID 5) inserted at the EcoRI site. The *cbh1* promoter is
 operably linked to the full length *egl1* cDNA (SEQ ID 6) coding sequence and to the

cbh1 transcriptional terminator (SEQ ID 7). The plasmid pMQ102 was digested with DraIII and BamHI and the released 280 bp fragment containing *hfb1* and linker sequences was purified from agarose gel and ligated to pPLE3 digested with DraIII and BamHI. The plasmid pMQ111 was digested with SacI and BamHI and the 260 bp
 5 fragment containing the *hfb1* sequence was ligated to pPLE3 digested with SacI and BamHI. The resulting plasmids pMQ103 (Figure 1) and pMQ113 (Figure 2) carry the coding sequences for full-length EGI linked to HFBI via a peptide linker and for EGICore linked to HFBI via its own linker region, respectively, under the control of *cbh1* promoter and terminator sequences.

10

Trichoderma expression vectors for production of EGI-HFBI and EGICore-HFBI fusion proteins under the control of *gpd1* promoter and terminator sequences of *Trichoderma* and *gpdA* promoter and *trpC* terminator sequences of *Aspergillus* were constructed as follows. A SacII site was inserted inbetween the XbaI and PacI sites of pMV4 using as
 15 an adapter annealed primers TAA CCG CGG T (SEQ ID 8) and CTA GAC CGC GGT TAA T (SEQ ID 9). The resulting plasmid is pMVQ. pMV4 contains a pNEB193 (New England Biolabs) backbone, and carries a 1.2 kb *Trichoderma gpd1* promoter (SEQ ID 10) and a 1.1 kb *gpd1* terminator (SEQ ID 11) inserted at Sall-XbaI and BamHI-AscI sites, respectively. The expression cassettes for EGI-HFBI and EGICore-HFBI were
 20 released from pMQ103 and pMQ113 with SacII and BamHI, purified from agarose gel and ligated to pMVQ cut with SacII and BamHI. The resulting plasmids pMQ104 (Figure 3) and pMQ114 (Figure 4) carry the EGI-HFBI and EGICore-HFBI cassettes, respectively, under the control of *Trichoderma gpd1* transcriptional control sequences. Expression plasmids pMQ105 (Figure 5) and pMQ115 (Figure 6) containing EGI-HFBI
 25 and EGICore-HFBI cassettes, respectively, operably linked to the *gpdA* promoter and *trpC* terminator of *Aspergillus* were constructed. EGI-HFBI and EGICore-HFBI cassettes were released from plasmids pMQ104 and pMQ114 with XbaI and BamHI, blunted with T4 DNA polymerase and ligated to NcoI digested and T4 DNA polymerase treated pAN52-1 (SEQ ID 12). pAN52-1 contains a pUC18 backbone, and
 30 carries a 2.3 kb *gpdA* promoter and a 0.7 kb *trpC* terminator sequences of *A. nidulans*.

Example 2

Construction of vectors for over-production of HFBI on cellulase-inducing and -repressing media

5 For over-expression of HFBI under *cbh1* promoter the protein coding region of *hfb1* was amplified with PCR using as a template pEA10 (Nakari-Setälä *et al.*, 1996). pEA10 carries a 5.8 kb genomic *Sall* fragment containing *hfb1* coding and flanking sequences. **GTC AA C CGC GG A CTG CGC ATC ATG AAG TTC TTC GCC ATC** (SEQ ID 13) was used as a 5' primer in the PCR and as a 3' primer SEQ ID 3. The sequence
10 in bold in the 5' primer is 21 bp of *cbh1* promoter adjacent to translational start site of the corresponding gene and the underlined CCGCGG is a *KspI* site. The obtained fragment of 430 bp was digested with *KspI* and *BamHI* and ligated to pMQ103 digested with *KspI* and *BamHI*. The resulting plasmid pMQ121 (Figure 7) carries the coding sequence of *hfb1* operably linked to *cbh1* transcriptional control sequences. pEA10
15 plasmid is used for over-production of HFBI in cellulase-repressing conditions.

Example 3

Transformation of *Trichoderma* and purification of the EGI-HFBI and EGIcore-HFBI producing and HFBI over-producing clones

20 *Trichoderma reesei* strains QM9414 (VTT-D-74075) and Rut-C30 (VTT-D-86271) were co-transformed essentially as described (Penttilä *et al.*, 1987) using 3–13 µg of the plasmids pMQ103, pMQ113, pMQ104, pMQ114, pMQ105, pMQ115, pMQ121 and pEA10 and as the selection plasmids 1–3 µg pToC202, p3SR2 or pARO21. pToC202
25 (pUC19 backbone) and p3SR2 (pBR322 backbone) plasmids carry 2.7 kb *XbaI* and 5 kb *EcoRI*-*Sall* genomic fragments of *A. nidulans*, respectively, containing the *amdS* gene (Hynes *et al.*, 1983; Tilburn *et al.*, 1983). pARO21 is essentially the same as pRLMex30 (Mach *et al.* 1994) and carries the *E. coli hph* gene operably linked to 730 bp of *pki1* promoter and 1 kb of *cbh2* terminator sequences of *T. reesei*. The Amd⁺ and
30 Hyg⁺ transformants obtained were streaked three times onto plates containing acetamide and hygromycin, respectively (Penttilä *et al.*, 1987). Thereafter spore suspensions were made from transformants grown on Potato Dextrose agar (Difco).

The production of the fusion proteins EGI-HFBI and EGICore-HFBI and HFBI was tested by slot blotting or Western analysis with EGI and HFBI specific antibodies from shake flask or microtiter plate cultivations carried out in minimal medium supplemented with either glucose, lactose or a mixture of Solka flock cellulose and/or spent grain and/or whey. The spore suspensions of the fusion protein producing clones were purified to single spore cultures on selection plates (containing either acetamide or hygromycin). To determine the best producers, production of the fusion proteins was analyzed again from these purified clones as described above.

- 10 *T. reesei* strains selected for further fermentor cultivations are VTT-D-98692 (pEA10), VTT-D-98492 (pMQ121), VTT-D-98693 (pMQ103), VTT-D-98691 (pMQ113), VTT-D-98681 (pMQ105) and VTT-D-98682 (pMQ115). These strains have QM9414 as the host strain. VTT-D-99702 (pMQ113) has Rut-C30 as the host strain.

15 **Example 4**

Cultivation of the EGI-HFBI and EGICore-HFBI protein producing and HFBI over-producing *Trichoderma* strains

- EGI-HFBI and EGICore-HFBI fusions were produced under the *cbh1* promoter in a 15-litre fermenter using *T. reesei* strains VTT-D-98693 (pMQ103) and VTT-D-98691 (pMQ113), respectively. Strains were grown 5 days on minimal medium (Penttilä *et al.*, 1987) containing 4% Solka flock cellulose (James River Corporation, Berlin, NH) and 2 % spent grain (Primalco, Koskenkorva, Finland). EGICore-HFBI was also produced in fermenter (15 l) using the Rut-C30 strain VTT-D-99702 (pMQ113) with 4 % lactose medium. To induce the production of EGI-HFBI and EGICore-HFBI fusions under *Aspergillus gpdA* promoter, *T. reesei* strains VTT-D-98681 (pMQ105) and VTT-D-98682 (pMQ115) were cultivated in 15-litre fermenter. Strains were grown 3 to 5 days on minimal medium supplemented with 2% glucose, 0.2% Peptone, and 0.1% Yeast Extract, and with glucose feed to maintain the glucose concentration in the range of 1 to 3% throughout the cultivation. HFBI over-producing strain VTT-D-98692 (pEA10) was grown similarly in 15 l on glucose medium and the strain VTT-D-98492 (pQM121) over-producing HFBI under *cbh1* promoter was cultivated for 7 days in 15-litre fermentor on medium containing 4% Solka flock and 2% spent grain. The control

cultivations with the host strains of the transformants, QM9414 (VTT-D-74075) and Rut-C30 (VTT-D-86271), were carried out on media containing i) Solka flock cellulose and either spent grain or whey, ii) lactose and iii) glucose similarly as described above.

5

When proper some *T. reesei* transformant strains and their host strains were also cultivated at 28°C in shake flasks for 5 to 6 days in 50 to 150 ml volume of *Trichoderma* minimal medium (Penttilä *et al.*, 1987) supplemented with either 3% Solka flock cellulose and 1% spent grain or 3–4% glucose with glucose feeding.

10

Example 5

Standard separation assays and analysis

If not otherwise stated the standard ATPS and subsequent analyses and calculations were carried out as explained in this example.

15

In general whole fermentation broth, supernatant (biomass separated by centrifugation or filtration) or purified proteins in buffer were separated in 10 ml graduated tubes. First detergent was added into the tubes and the tubes were then filled to 10 mg with protein containing liquid. The amount of detergent in the tube was calculated in weight percent of detergents. After thorough mixing in an overhead shaker the separation took place by either gravity settling in a water bath at constant temperature or by centrifugation at constant temperature. The separation usually was performed at 30°C, the standard amount of detergent used was 2–5% (w/v). After separation the volume ratio was noted and samples were taken from the lighter and heavier phase for analysis.

20

25

Two-phase separations were analysed qualitatively by using SDS-PAGE gels followed by visualization of the fusion proteins with Coomassie brilliant blue R-250 (Sigma) or Western blotting. Polyclonal anti-HFBI antibody were used in Western analysis for detection of EGICore-HFBI, EGI-HFBI and dCBD-HFBI proteins together with alkaline phosphatase conjugated anti-rabbit IgG (Bio-Rad). Alkaline phosphatase activity was detected colorimetrically with BCIP (5-bromo-4-chloro-3-indolyl-phosphate) used in conjunction with NBT (nitro blue tetrazolium) (Promega).

30

Contaminating endogenous EGI, CBHI and EGIII in the top phase was tested with appropriate antibodies. Acidic protease activity in the top and bottom phase was also tested using the SAP method (Food Chemicals Codex, p. 496–497, 1981), which is based on the 30 min enzymatic hydrolysis of a hemoglobin substrate. All reactions were performed at pH 4.7 and 40 °C. Unhydrolyzed substrate was precipitated with 14% TCA and removed by filtration. The released tyrosine and tryptophan was determined spectrophotometrically. Total protein concentrations were determined by *Non-Interfering Protein Assay*[™] (Geno Technology, Inc).

EGI activity was detected using 4-methylumbelliferyl-β-D-cellobioside (MUC) (Sigma M 6018) as substrate (Van Tilbeurgh H. & Caeysens M., 1985; Van Tilbeurgh *et.al.*, 1982). EGI hydrolyses the β-glycosidic bond and fluorogenic 4-methylumbelliferone is released, which can be measured using a fluorometer equipped with a 360 excitation filter and a 455 nm emission filter. CBHI also hydrolyses the substrate and it was inhibited by addition of cellobiose (C-7252, Sigma). EGI containing liquid was added in an appropriate dilution to a buffer containing 50 mM sodium acetate buffer (pH 5), 0.6 mM MUC and 4.6 mM cellobiose. The mixture was heated to 50°C. The reaction was stopped after ten minutes using 2% Na₂CO₃, pH 10. Purified CBHI was detected using the same assay as for EGI without the addition of the inhibitor cellobiose.

20

The partition coefficient K was defined as the ratio of the measured concentrations or activities in the top and bottom phase, respectively.

The Yield Y was defined as follows:

$$Y_T = \frac{1}{1 + \left[\frac{V_B}{V_T} \cdot \frac{1}{K} \right]}$$

25

where Y_T is the Yield of the top phase, V_B and V_T are the volumes of top and bottom phase, respectively. The Yield of the bottom phase can be described accordingly.

The mass balances, e.g. recovery of all added protein, were always checked for completeness to ensure no artificially high Yield (e.g. due to possible inactivation of the protein in the bottom phase). The values were usually calculated based on total enzyme

30

activity (EGI wt plus the EGI-fusion) and thus the values are underestimated for the separation of the fusion as demonstrated in Example 16.

Example 6

5 Small scale ATPS separation studies and gel analysis

EGI-HFBI and EGICore-HFBI fusions produced under the *cbh1* promoter in a 15-litre fermenter on Solka flock cellulose and spent grain medium as described in Example 4 using *T. reesei* strains VTT-D-98693 (pMQ103) and VTT-D-98691 (pMQ113),
10 respectively, were separated in small scale ATPS as described above.

The phases from the two phase separations were analysed qualitatively by using SDS-PAGE gels followed by visualization of the fusion proteins with Coomassie brilliant blue or Western blotting. Coomassie stained SDS-PAGE (10%) is shown in Figure 8. In the
15 lane containing the non-extracted culture filtrate three distinct closely migrating bands can be seen (the sample was diluted 1/10 with H₂O). The topmost band is CBHI, the band in the middle is EGICore-HFBI fusion and the lower one endogenous EGI. In the samples separated in ATPS, only two bands (CBHI and EGI) are seen in the sample from bottom phase and one band representing EGICore-HFBI in the sample obtained
20 from the top phase.

Western blotting with HFBI antibody showed thick bands for the top phase, whereas for the bottom phase there was only faint band demonstrating that the fusion is separating strongly into the detergent top phase. Figure 9 shows the separation of the EGICore-HFBI
25 HFBI fusion produced on cellulose media into the top phase. Contaminating endogenous EGI and EGIII in the top phase was tested with appropriate antibodies but no signal was detected.

Small amount of endogenous CBHI was found in the upper phase when CBHI antibody
30 was used in Western blotting. EGI, EGIII and proteases were not found in the top phase. Further purification from the contaminating CBHI was observed when the top phase was re-extracted with 2 % detergent. The Figure 10 shows that the upper phase does not any more contain CBHI and pure fusion protein is recovered.

EGIcore-HFBI was also produced in fermenter (15 l) using the Rut-C30 strain VTT-D-99702 (pMQ113) with 4 % lactose medium. The separation in ATPS carried out in the standard manner gave essentially the same result as the separation from cellulose containing medium thus demonstrating that the purification can be carried out from several media relevant for large scale industrial use.

Acidic protease activity in the top was only 1/15 compared to the bottom phase (table below) demonstrating that acidic proteases remain in the bottom phase.

	A (275 nm)	HUT ³ /ml
Bottom phase ¹	0.146	41.6
Top phase ²	0.009	2.6

¹1/10 diluted bottom phase after separation of VTT-D-98691 culture filtrate with 2% detergent

²1/100 diluted bottom phase after separation of VTT-D-98691 culture filtrate with 2% detergent

³1 HUT = enzyme concentration, which in reaction conditions hydrolyses hemoglobin in 1 min so that the absorbance at 275 nm of the formed hydrolysate equals 1.10 μ g tyrosine/ml 0.006 N HCl solution.

These results show that the fusion protein can be purified extremely efficiently and the resulting preparate is free of other proteins produced by the fungus including proteases.

Example 7

Recovery of the native EGI in ATPS after thrombin cleavage

EGI-HFBI protein produced by the strain VTT-D-98693 has a thrombin cleavage site (LVPRGS) designed in the linker region between the EGI CBD and HFBI, which would enable the recovery of the native EGI after thrombin cleavage. EGI-HFBI fusion protein was purified from the culture filtrate (100 ml) of strain VTT-D-98693 grown on 4% Solka flock cellulose and 2 % spent grain as described in Example 4 using the 2-phase separation system (5 % detergent). After removal of the bottom phase the detergent phase was extracted by isobutanol. The resulting water phase (~19 ml) was divided in eppendorf tubes and the liquid was evaporated with speed vac. Remaining lyophilizate

was diluted to 50 mM Tris-Cl (pH 8). To test the efficiency of thrombin cleavage, 9 units of thrombin (Sigma) was incubated > 24 h with 1 mg EGI-HFBI fusion protein in 36 °C at pH 8.0. Coomassie stained SDS-PAGE (10 %) was used for detection.

- 5 Only minor cleavage was observed in 48 h under these conditions (Figure 11), possibly due to steric hindrance by O-glycosylation in the linker.

Example 8

Separation of low concentrations of EGICore-HFBI in ATPS

10

Detergent based aqueous two-phase systems were successfully applied using very low concentrations (diluted) of EGICore-HFBI fusion protein produced with the *cbh1* promoter in *T. reesei* VTT-D-98691 (pMQ 113) from a 15 liter cultivation carried out on Solka flock cellulose with spent grain as described in Example 4.

15

The original protein concentration of the supernatant was 7.0 mg/ml. This supernatant was diluted with de-ionised water by a factor of 100 and 1000, respectively. The fusion protein could be separated using 2% (w/w) of the detergent C12-C18EO5 with partitioning coefficients higher than 5. This is shown in the table below together with the experiment with non-diluted supernatant. The partitioning coefficients were calculated based on activity measurements for total EGI (wild type and fusion protein together).

20

	EGICore-HFBI non-diluted supernatant	dilution 1/100	dilution 1/1000
25 K	4.1	5.3	5.6
Y [%]	38	31	32

Example 9

Separation of EGCore-HFBI from fungal biomass containing culture broths

EGCore-HFBI from *T.reesei* strain VTT-D-98691 (pMQ 113-2) was cultivated (50
5 ml in 250 ml shake flasks) on Solka flock cellulose with spent grain as described in
Example 4. Directly after the cultivation, part of the whole broth was centrifuged at
3000 rpm for 30 min, supernatant was spilled out and the centrifuged mycelium was
added to the supernatant to obtain artificial whole broths containing different amounts
of biomass.

10

Using 5% of C12-C18EO5 in a 10 g experiment consisting of up to 50% wet biomass
(weight of wet biomass divided by the sum of wet biomass and supernatant) could still
be separated without any difficulties. The Yield remained in between 61 and 64 % and
therefore it is not significantly different in comparison to the experiment carried out with
15 supernatant only (without mycelium) (see table below). The total recovery of the
fusion protein is even higher. This is most probably due to cell attached enzyme
extracted in the ATPS increasing the total amount of EGI. The partitioning coefficient
was calculated based on activity measurements for total EGI (wild type and fusion
protein together).

20

	K	Y[%]
supernatant	5.5	62
25% of wet biomass in supernatant	7.3	66
25 40% of wet biomass in supernatant	6.4	61
50% of wet biomass in supernatant	7.6	64

30

Example 10**Separation of EGI-HFBI in ATPS**

5 EGI-HFBI from *Trichoderma reesei* strain VTT-D-98693 (pMQ 103) from a 15 liter cultivation carried out on Solka flock cellulose and spent grain as described in Example 4 was separated in a 10 g experiment using different amounts of C12-C18EO5. The partitioning coefficients are shown below. The partitioning coefficient was calculated based on activity measurements for total EG1 (wild type and fusion protein together), and as in previous examples the endogenous EGI is included in the partitioning

10 coefficients.

Detergent [% w/w]	2	3	5	7
K	1.9	1.8	1.4	1.1

15

Example 11**Separation of EGICore-HFBI in 50 ml**

20 EGICore-HFBI from *T. reesei* strain VTT-D-98691 (pMQ 113) cultivated in 15 liters using Solka flock cellulose and spent grain as described in Example 4 was separated in Falcon tubes in a 50 g experiment using 5% of C12-C18EO5. A partition coefficient of 2.52 and a yield of 51 % could be obtained. The separation was performed at 30°C at 3000rpm for 30 minutes. The values are based on activity measurements for total EGI activity (wild type and fusion protein together) including endogenous EGI.

25

Example 12**Separation of EGICore in ATPS using different detergents**

30 EGICore-HFBI from *T. reesei* strain VTT-D-98691 (pMQ 113) cultivated in 15 liters using Solka flock cellulose with spent grain as described in Example 4 was separated in a 10 g experiment using 2% of detergent in each experiment. The detergents investigated in this example were C10 EO5, C12 EO5, C14 EO6 (each Nikko

Chemicals, Japan), C12–C18 EO5 („Agrimul NRE 1205“, Henkel, Germany), C12/14 5EO, C12/14 6EO (Clariant, Germany), C9/11 EO5.5 („Berrol 266“, Akzo Nobel, Germany), Triton X–114 (Sigma, Germany). The partition coefficients and yields are listed below. The values are based on activity measurements for total EGI activity (wild type and fusion protein together) including endogenous EGI.

	K	Y(fusion) [%]
C10EO5	20	56
C12EO5	15	57
C12–C18EO5	14	66
C12/14 5EO	12	58
C12/14 6EO	14	62
C14EO6	11	54
C9/11 EO5.5	5	30
Triton X–114	0.16	53

Example 13

Separation of EGICore–HFBI in ATPS from glucose grown cultures

EGICore–HFBI was separated from cultivation of the *Trichoderma reesei* strain VTT–D–98682 (pMQ115) cultivated with glucose as described in Example 4. The supernatant was separated with 2% of the detergent C12–C18 EO5. The fusion protein could be partitioned with a K value of 2.4. In comparison, the K value for the native EGI is 0.3 when measured in a similar way for purified EGI.

Example 14

Separation of EGICore–HFBI using different concentrations of detergent

EGICore–HFBI from *T. reesei* VTT–D–98691 (pMQ 113) cultivated in 15 litres using Solka flock with spent grain as described in Example 4 was separated in detergent based ATPS applying different amounts of the detergent C12–C18 EO5 on the cell free

supernatant. The partitioning coefficients are shown in the table below. The corresponding gel electrophoresis and Western antibody-blot are shown in Figure 8 and Figure 9, respectively.

The values are based on activity measurements of total EGI activity.

5	Amount of detergent C12-C18 EO5	K	Yield(%)
	1.0%	6.1	9
	2.0%	4.1	38
	3.5%	3.6	50
	5.0%	2.9	55
10	7.5%	1.7	53
	10.0%	1.1	58

Example 15

15 Re-extraction of the detergent phase

Detergent based ATPS was applied on EGICore-HFBI fusion protein containing supernatant produced by the strain VTT-D-98691 (pMQ 113) in a shake flask cultivation. The first extraction using C12-C18EO5 conducted under the standard conditions shows a partitioning coefficient of 16 and a yield of 72 % (wild type EGI measured together with fusion protein). The top phase was re-extracted in 10 mM sodium acetate buffer (pH 5) with 2% of detergent. A partitioning coefficient of 52 and a yield of 89 % could be obtained. In the re-extraction experiment of the bottom phase (2% of detergent), a small yield of 7.5% and a K of 0.8 of EGI activity were achieved. The partitioning coefficients were calculated based on activity measurements for total EGI (wild type and fusion protein together). Due to the wild type EGI present in the sample, the yield is at least 72% and the partitioning coefficient at least 16 in the first extraction. The SDS-PAGE results of both extractions are shown in Figure 10.

30	Separation step	K	Y[%]
	2% detergent	16	72
	reextraction top phase	52	89
	reextraction bottom phase	0.8	7.5

Example 16**Separation of pure cellulases in ATPS**

The effect of HFBI on partitioning and the final yield can further be demonstrated by comparing the extraction result of EGIcore-HFB fusion with extraction results obtained with purified wild type EGI and EGIcore. The fusion protein is partitioning more than 100 times better to the detergent phase (see table below).

The improvement on the partitioning of the purified fusion protein from the first extraction obtained in the re-extraction (see Example 15) can be explained by the partitioning of the wild type EGI as demonstrated with purified wild type EGI in the table below. The wild type EGI lowers the partitioning coefficient in the first extraction (since EGI activity is measured from both top and bottom phase), but the absence of it in the re-extraction increases the partitioning coefficient of the EGIcore-HFBI fusion. The purity can in addition be demonstrated by analysing the partitioning of pure CBHI, which is the major contaminating protein corresponding to about 50 % of all secreted *T. reesei* proteins. Pure CBHI has a partitioning coefficient of 0.5 and a yield of 3.6 and is therefore separated from the fusion protein.

20

Separation step	K	Y[%]
re-extraction of top phase	52	89
extraction of pure wild type EGI	0.3	2.2
extraction of pure EGI-core	0.3	2.3
extraction of pure CBHI	0.5	3.6

25

Using the definitions of K and Y and calculating mass balances; the ratio of the amount of EGI fusion protein to EGI wild type can be calculated. The "true" partition coefficients and Yields can be concluded from this. "True" means the values which would be detected if it would be possible to measure only the amount of EGI-fusion without measuring the amount for EGI wild type at the same time.

30

The fundament for the calculation is the re-extraction experiment. The re-extracted top phase is believed to be pure. An example of the measured values and the calculated "true" values based on this are shown in the table below for two cultivations of VTT-D-98691 (pMQ113) grown as described in Example 4.

5

10

cultivation vessel	cultivation substrate	K "with EG1 wt"	"true" K	Y [%] "with EG1 wt"	"true" Y [%]
15 liter fermenter	whey permeate	4	6	16	54
250 ml shake flask	cellulose	16	54	66	90

15

Example 17

HFBI and HFBII purification in ATPS

20

HFBI was produced by cultivating the *T. reesei* strain VTT-D-98692 (pEA10-103B) using glucose as substrate as described in Example 4. HFBI could be separated using 2% of the detergent C12-C18 EO5 with a partition coefficient higher than 20 under the standard conditions described.

25

HFBII was produced by cultivating the *T. reesei* strain VTT-D-74075 (QM9414) on whey spent grain as described in Example 4. HFBII could be separated using 2% of the detergent C12-C18 EO5, exceeding a partition coefficient of 10 under the standard conditions.

30

Both HFBI and HFBII hydrophobins are thus partitioning well to the upper phase in ATPS.

Example 18**Detergent based ATPS with additional NaCl**

EGIcore-HFBI from which cultivation of *T. reesei* was separated in a 10 g experiment
 5 using 5% of C12-C18EO5. The partitioning coefficient of the supernatant was 3.5 with
 a volume ratio of 0.2. Using 1.1 % (w/v) NaCl the partitioning coefficient could be
 increased to 4.3 with a lower volume ratio of 0.14.

Example 19

10 **Construction of an *E. coli* strain expressing a fusion protein HFBI-dCBD,
 containing hydrophobin I and double cellulose binding (CBD) domains**

A 280 bp DNA fragment containing a modified *cbh2* linker region followed by the
 coding region of *hfb1* from Ser-23 to the STOP codon was amplified by PCR using the
 15 plasmid pARO1 (Nakari-Setälä *et al.*, 1996) as a template. The 5' primer was 5' TCT
 AGC **AAG CTT** GGC TCT AGT TCT GGA ACC GCA CCA GGC GGC AGC
 AAC GGC AAC GGC AAT GTT TGC (SEQ ID 14) and the 3' primer was 5' TCG
 TAC **AAG CTT** TCA AGC ACC GAC GGC GGT (SEQ ID 15). The sequences in bold
 in the 5' and 3' primers encode the modified CBHII linker (Gly Ser Ser Ser Gly Thr
 20 Ala Pro Gly Gly) and a translational STOP, respectively, and the underlined AAGCTT
 in both primers is a HindIII site. The PCR fragment was purified from agarose gel,
 digested with HindIII and ligated to HindIII digested and SAP treated (Shrimp Alkaline
 Phosphatase, USB) pSP73 resulting in plasmid pTNS9.

25 For subsequent cloning of the modified CBHII linker-HFBI fragment to an *E. coli*
 expression vector, pTNS9 was digested with HindIII and the proper fragment was
 purified from agarose gel. This HindIII fragment was cloned to HindIII digested and
 SAP treated (Shrimp Alkaline Phosphatase, USB) B599 resulting in pTNS13 (Figure
 12). The *E. coli* expression vector B599 is essentially the same as the one described by
 30 Linder *et al.* (1996) except that it is missing a STOP codon at the end of the protein
 coding sequence. It carries the coding sequence for a fusion protein containing CBHII
 CBD (41 N-terminal residues of CBHII) and CBHI CBD linked together via CBHI
 linker region (CBHI linker and CBD are the last 57 residues in CBHI). The expression

and secretion of the fusion protein in B599 is under the control of *tac* promoter and *pelB* signal sequence (Takkinen *et al.*, (1991). pTNS13 expression vector thus carries the coding region for a fusion protein of double CBD and HFBI linked in frame via the Gly-Ser-Ser-Ser-Gly-Thr-Ala-Pro-Gly-Gly peptide. This vector also contains the
 5 *amp* gene for selection of *E. coli* transformants. pTNS13 plasmid was transformed into *E. coli* strain RV308 (*su*-, Δ *lacX74*, *galISII::OP308*, *strA*) and this strain was used for production of the fusion protein.

Example 20

10 Separation of HFBI-dCBD molecules expressed in *E.coli* in ATPS

dCBD-HFBI was produced in *E. coli* strain RV 308 transformed with pTNS13 plasmid as described above. The inoculum of RV308/pTNS13 was grown to the exponential growth phase in LB medium containing ampicillin (0.1 g/l) and 1% glucose.
 15 Fermentation was carried out using mineral salt medium described by Pack *et al.* (1993) with glucose (feed) in 10 litre fermenter. During cultivation temperature was maintained at 28 °C and pH was controlled at 6.8 with NH₄OH. Cell growth was monitored by measuring OD₆₀₀ and dry weight of biomass. The culture was induced with 50 µM (final concentration) IPTG (isopropyl-β-D-thiogalactopyranoside) at late-exponential growth
 20 phase (OD₆₀₀=50-60) to promote fusion protein production.

Two-phase separation analysis of dCBD-HFBI protein was performed using culture filtrate and 5% detergent in the total volume of 40 ml. Results from Western blotting showed that 2-phase separation with 5 % detergent in the standard way was highly
 25 specific also for the dCBD-HFBI fusion. Strong signal was observed in the sample from the detergent phase compared to the sample from the bottom phase as shown in Figure 13.

Example 21

Construction of yeast strains expressing HFBI-FloI fusion protein on the cell surface

5 For construction of a HFBI-FLO1 fusion protein expression cassette, *hfb1* (SEQ ID 1) coding region (from Ser-23 to the STOP codon) was amplified with PCR using pARO1 (Nakari-Setälä *et al.*, 1996) as a template and as a 5' primer TCT AGC TCT AGA AGC AAC GGC AAC GGC AAT GTT (SEQ ID 16) and as a 3' primer TGC TAG TCG ACC TGC TAG CAG CAC CGA CGG CGG TCT G (SEQ ID 17). The underlined sequences in the 5' and 3' primers are XbaI and NheI sites, respectively. The 0.225 bp PCR fragment was purified from agarose gel and ligated to pGEM-T vector (Promega) resulting in pTNS10. The *hfb1* fragment was released from pTNS10 with XbaI and NheI and ligated to pTNS15 cut with the same restriction enzymes. Plasmid pTNS15 (Figure 14) is essentially the same as plasmid pBR-ADH1-FLO1L by 10 Watari *et al.* 1994 except that a NheI site in the pBR322 backbone has been replaced by a BglII site and a unique XbaI site is introduced by linker cloning in the unique AocI site preceding the putative signal sequence cleavage site. The resulting plasmid pTNS18 (Figure 15) contains the complete expression cassette for HFBI-FLO1 fusion protein in which HFBI substitutes the putative lectin domain from Ser-26 to Ser-319 in the yeast 15 flocculin FLO1 (SEQ ID 18).

In the next step, yeast expression vector for production of HFBI-FLO1 fusion protein was constructed. The expression vector used as a backbone in the construct is pYES2 (Invitrogen) (SEQ ID 19) which is a high-copy episomal vector designed for inducible 25 expression of recombinant proteins in *S. cerevisiae*. It carries *GAL1* promoter and *CYC1* terminator sequences which regulate transcription, and 2μ origin of replication and *URA3* gene for maintenance and selection in the host strain. The plasmid pTNS18 was digested with HindIII and the released 3.95 kb fragment containing the expression cassette for HFBI-FLO1 was purified from agarose gel and ligated to pYES2 digested with HindIII. 30 This ligation mixture was concentrated by standard ethanol precipitation. The ligation mixture should contain besides unligated fragments and uncorrect ligation products also molecules where the vector and insert are correctly ligated with each other to result in

plasmid pTNS23 (Fig. 16) which carries the expression cassette for HFBI-FLO1 operably linked to *GAL1* and *CYC1* terminator sequences.

The above ligation mixture was transformed using the LiAc method of Gietz *et al.* (1992) into a laboratory *S. cerevisiae* strain H452 (wild type W303-1A; Thomas and Rothstein, 1989). Transformant colonies able to grow on SC-URA plates were picked and streaked on selective plates. Nitrocellulose replicas were taken from the plates and treated for colony hybridization according to Sherman *et al.* (1983). To find those yeast colonies containing the pTNS23 plasmid, replicas were hybridized with digoxigenin labelled *hfb1* coding fragment after which an immunological detection was performed all according to the manufacturer (Boehringer Mannheim). Plasmids were recovered from several yeast colonies giving positive hybridization signal by isolating total DNA and using this in electroporation of *E. coli*. Restriction mapping and sequencing were carried out to confirm that the pTNS23 plasmid in the yeast transformants was correct. One of the transformants carrying plasmid pTNS23 was chosen for further studies and was designated VTT-C-99315. The control strain for it is yeast strain H2155 which carries the plasmid pYES2 in H452 background.

Example 22

20 Separation of yeast cells expressing HFBI-FloI fusion protein in ATPS

The *Saccharomyces cerevisiae* strain VTT-C-99315 (vector pTNS23) and its control strain H2155 (vector pYES2) were cultivated on synthetic complete medium lacking uracil (SC-URA) (Sherman, 1991) with 2% galactose as the carbon source to give an A_{600} of approximately 4. Approximately 6.3×10^8 cells in their culture medium were taken to ATPS using 7% (w/v) C12-18EO5 detergent (Agrimul NRE from Henkel) in a total volume of 5 ml. ATPS was carried out using standard protocol. After phase separation by gravity settling, the top detergent phase was clearly turbid in the case of the strain VTT-C-99315 in contrast to the control strain whose detergent phase was clear (Figure 17). Samples were taken from the top phases and dilution series from 10^{-1} to 10^{-5} were prepared in 0.9% NaCl and plated on YPD plates. After incubation at 30°C the amount of yeast colonies were calculated showing at least 70 times more yeast colonies of the strain VTT-C-99318 on YPD plates compared to the control strain. This

clearly manifests that also in a system over-loaded with cells, separation to the detergent phase of cells expressing a hydrophobin on cell surface occurs.

Example 23

5 **Production of EGICore-HFBI fusion proteins in *T. reesei* $\Delta hfb2$ strain for improved partitioning of the fusion protein in ATPS**

Trichoderma reesei strain QM9414 $\Delta hfb2$ (VTT-D-99726) was transformed essentially as described (Penttilä *et al.*, 1987) using 10 μ g of the plasmid pMQ113 together with
 10 3 μ g of the selection plasmid pTOC202 containing the *amdS* gene (Hynes *et al.*, 1983); Tilburn *et al.*, 1983) of *Aspergillus nidulans* encoding for acetamidase. pMQ113 contains an expression cassette for production of EGICore-HFBI fusion protein under the control of *cbh1* promoter and terminator sequences.

15 The Amd+ transformants obtained were streaked two times onto plates containing acetamide (Penttilä *et al.*, 1987). Thereafter spore suspensions are made from transformants grown on Potato Dextrose agar (Difco). The production of the EGICore-HFBI fusion protein is tested by slot blotting or Western analysis with EGI and HFBI specific antibodies from shake flask or microtiter plate cultivations carried out in
 20 minimal medium supplemented with a mixture of Solka flock cellulose and/or spent grain and/or whey. The spore suspensions of the clones producing fusion protein are purified to single spore cultures on selection plates (containing acetamide). To determine the best producers, production of the fusion protein is analyzed again from these purified clones as described above.

25

For partitioning experiments of the EGICore-HFBI fusion protein in ATPS using the polyoxyethylene detergent C₁₂₋₁₈EO₅ (Agrimul NRE 1205, Henkel) the best production strain obtained in this study and as control strains VTT-D-98691 (QM9414 strain producing EGICore-HFBI) and VTT-D-74075 (QM9414) are cultivated at 28°C in
 30 shake flasks for 5 to 6 days in 50 to 250 ml volume of *Trichoderma* minimal medium (Penttilä *et al.*, 1987) supplemented with 3% Solka flock cellulose and 1% spent grain.

Standard partitioning experiments as described in Example 5 are carried out with culture supernatants (biomass separated by centrifugation or filtration). After separation the volume ratio of the lighter and heavier phase is noted and the concentration factor for the fusion protein is calculated from it. Samples are also taken from the lighter and heavier phase and analysed with SDS-PAGE, Western blotting and activity measurements as described in Example 5. Partition coefficients (K) and yields (Y) are calculated as described in Example 5.

Example 24

10 Production of HFBI-single chain antibody fusion protein in *T. reesei* $\Delta hfb2$ strain for purification in ATPS

A *T. reesei* strain is constructed which produces a fusion protein consisting of *T. reesei* HFBI protein in the N-terminus and in the C-terminus a single chain antibody recognizing a small molecular weight derivative of diarylalkyltriazole (ENA5SCFV). Production of the fusion protein is under the *cbh1* regulatory sequences. The fusion is to be subjected for further purification using aqueous two-phase system.

For construction of HFBI-ENA5SCFV fusion protein, pENA5SCFV was digested with NcoI and XbaI. The fragment containing the *ena5scfv* gene and the histidine tail (6 x His) was blunt-end cloned to pTNS29 resulting in PTH1. pENA5SCFV vector carries the coding region for ENA5 single chain antibody consisting of the variable domains of the heavy and light chains connected via a glycine serine linker and a 6 x histidine tag at the C-terminal end. Transcription and secretion of the single chain antibody are under control of the *tac* promoter and *pelB* signal sequence, respectively (Takkinen *et al.*, 1991). pTNS29 vector carries the *hfb1* coding region of *T. reesei* followed by a linker sequence (ProGlyAlaSerThrSerThrGlyMetGlyProGlyGly) under the control of *cbh1* promoter and terminator sequences.

For construction of HFBI-ENA5SCFV fusion protein with a thrombin cleavage site in the linker peptide, *ena5scfv* coding region (from Ala-23 to the STOP codon) and a peptide linker containing the thrombin cleavage site (Gly Thr Leu Val Pro Arg Gly Pro Ala Glu Val Asn Leu Val) preceeding it was amplified with PCR using pENA5SCFV

as a template and as a 5' primer GAA TTC GGT ACC *CTC GTC CCT CGC GGT CCC*
 GCC GAA GTG AAC CTG GTG and as a 3' primer TGA ATT CCA TAT GCT AAC
 CCC GTT TCA TCT CCA G. The sequence in bold in the 5' primer encodes the first
 6 N-terminal residues of ENA5SCFV. The sequence in italics is a thrombin cleavage
 5 site and underlined GGT ACC is Asp718 site. The sequence in bold in the 3' primer
 encodes the 6 C-terminal residues of ENA5SCFV and the underlined CA TATG is a
 NdeI site. The 790 bp PCR fragment was purified from agarose gel and ligated to
 pTNS29 resulting in pTH2.

10 *Trichoderma reesei* strain VTT-D-99726 (QM9414 $\Delta hfb2$) is co-transformed essentially
 as described (Penttilä *et al.*, 1987) using 10 μ g of the plasmids pTH1 and PTH2 and as
 selection plasmid 2 μ g pTOC202. Amd+ transformants obtained are streaked two times
 onto plates containing acetamide. Thereafter spore suspensions are made from
 transformants grown on Potato Dextrose agar (Difco).

15

The production of the two HFBI-ENA5SCFV fusion proteins is tested by Western
 analysis with HFBI specific antibody and with the antibody against the his-tail from
 shake flask cultivations carried out in minimal medium supplemented with 3 % lactose
 or Solka flock cellulose and spent grain.

20

Partitioning experiments of the HFBI-ENA5 fusion proteins in ATPS using the
 polyoxyethylene detergent C₁₂₋₁₈EO₅ (Agrimul NRE 1205, Henkel) with the supernatants
 of the best production strains obtained in this study, and the control strain VTT-D-
 99726 (QM9414 $\Delta hfb2$) are carried out and analysed as described in Example 5.

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Sequences

SEQ ID 1: Coding sequence of *hfb1* , 428 bp, introns underlined.

ATGAAGTT CTTCGCCATC GCCGCTCTCT TTGCCGCCGC
 TGCCGTTGCC CAGCCTCTCG AGGACCGCAG CAACGGCAAC GGCAATGTTT GCCCTCCCGG
 CCTCTTCAGC AACCCCCAGT GCTGTGCCAC CCAAGTCCTT GGCCTCATCG GCCTTGACTG
 CAAAGTCCGT AAGTTGAGCC ATAACATAAG AATCCTCTTG ACGGAAATAT GCCTTCTCAC
 TCCTTTACCC CTGAACAGCC TCCCAGAACG TTTACGACGG CACCGACTTC CGCAACGTCT
 GCGCCAAAAC CGGCGCCCAG CCTCTCTGCT GCGTGGCCCC CGTTGTAAGT TGATGCCCCA
 GCTCAAGCTC CAGTCTTTGG CAAACCCATT CTGACACCCA GACTGCAGGC CGGCCAGGCT
 CTTCTGTGCC AGACCGCCGT CGGTGCTTGA

SEQ ID 2

TCG GG C ACT ACG TG C CAG TAT AGC AAC GAC TAC TAC TCG CAA TGC CTT GTT
 CCG CGT GGC TCT AGT TCT GGA ACC GCA

SEQ ID 3

TCG TAC GGA TCC TCA AGC ACC GAC GGC GGT

SEQ ID 4

ACT ACA CGG AG G AGC TC G ACG ACT TCG AGC AGC CCG AGC TGC ACG CAG AGC
 AAC GGC AAC GGC

SEQ ID 5: *T. reesei cbhl* promoter, 2211 bp

GAATTCTCAC GGTGAATGTA GGCCTTTTGT AGGGTAGGAA TTGTCACTCA AGCACCCCCA
 ACCTCCATTA CGCCTCCCC ATAGAGTTCC CAATCAGTGA GTCATGGCAC TGTTCTCAA
 TAGATTGGGG AGAAGTTGAC TTCCGCCAG AGCTGAAGGT CGCACAACCG CATGATATAG
 GGTGCGCAAC GGCAAAAAG CACGTGGCTC ACCGAAAAGC AAGATGTTTG CGATCTAACA
 TCCAGGAACC TGGATACATC CATCATCAG CACGACCACT TTGATCTGCT GGTAAACTCG
 TATTCGCCCT AAACCGAAGT GCGTGGTAAA TCTACACGTG GGCCCCCTTC GGTATACTGC
 GTGTGTCTTC TCTAGGTGCA TTCTTTCCTT CCTCTAGTGT TGAATTGTTT GTGTTGGGAG
 TCCGAGCTGT AACTACCTCT GAATCTCTGG AGAATGGTGG ACTAACGACT ACCGTGCACC
 TGCATCATGT ATATAATAGT GATCCTGAGA AGGGGGGTTT GGAGCAATGT GGGACTTTGA
 TGGTCATCAA ACAAAGAACG AAGACGCCCT TTTTGCAAAG TTTTGTTTCG GCTACGGTGA
 AGAACTGGAT ACTTGTTGTG TCTTCTGTGT ATTTTGTGG CAACAAGAGG CCAGAGACAA
 TCTATTCAA CACCAAGCTT GCTCTTTTGA GCTACAAGAA CCTGTGGGGT ATATATCTAG
 AGTTGTGAAG TCGGTAATCC CGCTGTATAG TAATACGAGT CGCATCTAAA TACTCCGAAG
 CTGCTGCGAA CCCGGAGAAT CGAGATGTGC TGGAAAGCTT CTAGCGAGCG GCTAAATTAG
 CATGAAAGGC TATGAGAAAT TCTGGAGACG GCTTGTGAA TCATGGCGTT CCATTCTTCG

ACAAGCAAAG	CGTTCGGTCG	CAGTAGCAGG	CACTCATTCC	CGAAAAAACT	CGGAGATTCC
TAAGTAGCGA	TGGAAACCGGA	ATAATATAAT	AGGCAATACA	TTGAGTTGCC	TCGACGGTTG
CAATGCAGGG	GTAAGTACCT	TGGACATAAC	TGTTCCGTAC	CCCACCTCTT	CTCAACCTTT
GGCGTTTCCC	TGATTCAGCG	TACCCGTACA	AGTCGTAATC	ACTATTAACC	CAGACTGACC
GGACGTGTTT	TGCCCTTCAT	TTGGAGAAAT	AATGTCATTG	CGATGTGTAA	TTTGCCTGCT
TGACCGACTG	GGGCTGTTCG	AAGCCCGAAT	GTAGGATTGT	TATCCGAACT	CTGCTCGTAG
AGGCATGTTG	TGAATCTGTG	TCGGGCAGGA	CACGCCTCGA	AGGTTACCGG	CAAGGGGAAAC
CACCGATAGC	AGTGTCTAGT	AGCAACCTGT	AAAGCCGCAA	TGCAGCATCA	CTGGAAAAATA
CAAACCAATG	GCTAAAAGTA	CATAAGTTAA	TGCCTAAAGA	AGTCATATAC	CAGCGGCTAA
TAATTGTACA	ATCAAGTGGC	TAAACGTACC	GTAATTTGCC	AACGCGTTGT	GGGGTTGCAG
AAGCAACGGC	AAAGCCCACT	TCCCACGTTT	GTTTCTTCAC	TCAGTCCAAT	CTCAGCTGGT
GATCCCCCAA	TTGGGTTCGT	TGTTTGTTC	GGTGAAGTGA	AAGAAGACAG	AGGTAAGAAT
GTCTGACTCG	GAGCGTTTGT	CATACAACCA	AGGGCAGTGA	TGGAAGACAG	TGAAATGTTG
ACATTCAAGG	AGTATTTAGC	CAGGGATGCT	TGAGTGTATC	GTGTAAGGAG	GTTTGTCTGC
CGATACGACG	AATACTGTAT	AGTCACTTCT	GATGAAGTGG	TCCATATTGA	AATGTAAGTC
GGCACTGAAC	AGGCAAAAGA	TTGAGTTGAA	ACTGCCTAAG	ATCTCGGGCC	CTCGGGCTTC
GGCTTTGGGG	GTACATGTTT	GTGCTCCGGG	CAAATGCAAA	GTGTGGTAGG	ATCGACACAC
TGCTGCCCTT	ACCAAGCAGC	TGAGGGTATG	TGATAGGCAA	ATGTTACAGG	GCCACTGCAT
GGTTTCGAAT	AGAAAGAGAA	GCTTAGCCAA	GAACAATAGC	CGATAAAGAT	AGCCTCATT
AACGAAATGA	GCTAGTAGGC	AAAGTCAGCG	AATGTGTATA	TATAAAGGTT	CGAGGTCCGT
GCCTCCCTCA	TGCTCTCCCC	ATCTACTCAT	CAACTCAGAT	CCTCCAGGAG	ACTTGTACAC
CATCTTTTGA	GGCACAGAAA	CCCAATAGTC	AACCGCGGAC	TGCGCATCAT	G

SEQ ID 6: *T. reesei* *egl1* cDNA, 1588 bp

CCCCCTATC	TTAGTCCTTC	TTGTTGTCCC	AAAATGGCGC	CCTCAGTTAC	ACTGCCGTTG
ACCACGGCCA	TCCTGGCCAT	TGCCCCGGTC	GTCGCCGCC	AGCAACCGGG	TACCAGCACC
CCCAGAGTCC	ATCCCAAGTT	GACAACCTAC	AAGTGTAACA	AGTCCGGGGG	GTGCGTGGCC
CAGGACACCT	CGGTGGTCC	TGACTGGAAC	TACCGCTGGA	TGCACGACGC	AAACTACAAC
TCGTGCACCG	TCAACGGCGG	CGTCAACACC	ACGCTCTGCC	CTGACGAGGC	GACCTGTGGC
AAGAAGTCT	TCATCGAGGG	CGTCGACTAC	GCCGCCTCGG	GCGTCACGAC	CTCGGGCAGC
AGCCTCACCA	TGAACCAAGT	CATGCCCAGC	AGCTCTGGCG	GCTACAGCAG	CGTCTCTCCT
CGGCTGTATC	TCCTGGACTC	TGACGGTGAG	TACGTGATGC	TGAAGCTCAA	CGGCCAGGAG
CTGAGCTTCG	ACGTGACCT	CTCTGCTCTG	CCGTGTGGAG	AGAACGGCTC	GCTCTACCTG
TCTCAGATGG	ACGAGAACGG	GGGCGCCAAC	CAGTATAACA	CGGCCGGTGC	CAACTACGGG
AGCGGCTACT	GCGATGCTCA	GTGCCCCGTC	CAGACATGGA	GGAACGGCAC	CCTCAACACT
AGCCACCAAG	GCTTCTGCTG	CAACGAGATG	GATATCCTGG	AGGGCAACTC	GAGGGCGAAT
GCCTTGACCC	CTCACTCTTG	CACGGCCACG	GCCTGCGACT	CTGCCGGTTG	CGGCTTCAAC
CCCTATGGCA	GCGGCTACAA	AAGCTACTAC	GGCCCCGGAG	ATACCGTTGA	CACCTCCAAG
ACCTTCACCA	TCATCACCCA	GTTCAACACG	GACAACGGCT	CGCCCTCGGG	CAACCTTGTTG
AGCATCACCC	GCAAGTACCA	GCAAAACGGC	GTCGACATCC	CCAGCGCCCA	GCCCCGGGGC
GACACCATCT	CGTCTGCCC	GTCCGCCTCA	GCCTACGGCG	GCCTCGCCAC	CATGGGCAAG
GCCCTGAGCA	GCGGCATGGT	GCTCGTGTTC	AGCATTGGA	ACGACAACAG	CCAGTACATG
AACTGGCTCG	ACAGCGGCAA	CGCCGGCCCC	TGCAGCAGCA	CCGAGGGCAA	CCCATCCAAC
ATCCTGGCCA	ACAACCCCAA	CACGCACGTC	GTCTTCTCCA	ACATCCGCTG	GGGAGACATT
GGGTCTACTA	CGAAGTCCAG	TGCGCCCCCG	CCCCCGCCTG	CGTCCAGCAC	GACGTTTTTCG
ACTACACGGA	GGAGCTCGAC	GACTTCGAGC	AGCCCCAGCT	GCACGCAGAG	CTACTGGGGG
CAGTGCAGTG	GCATTGGGTA	CAGCGGGTGC	AAGACGTGCA	CGTCGGGCAC	TACGTGCCAG
TATAGCAACG	ACTACTACTC	GCAATGCCTT	TAGAGCGTTG	ACTTGCCTCT	GGTCTGTCCA
GACGGGGGCA	CGATAGAATG	CGGGCACGCA	GGGAGCTCGT	AGACATTGGG	CTTAATATAT
AAGACATGCT	ATGTTGTATC	TACATTAGCA	AATGACAAAC	AAATGAAAAA	GAACCTATCA
AGCAAAAAAA	AAAAAATAAA	AAAAAATA			

SEQ ID 7: *T. reesei* *cbh1* terminator, 745 bp

GGACCTACCC	AGTCTCACTA	CGGCCAGTGC	GGCGGTATTG	GCTACAGCGG	CCCCACGGTC
TGCGCCAGCG	GCACAAGTTG	CAGGTCCTG	AACCTTACT	ACTCTCAGTG	CCTGTAAAGC
TCCGTGCGAA	AGCCTGACGC	ACCGGTAGAT	TCTTGGTGAG	CCCGTATCAT	GACGGCGGGC
GGAGCTACAT	GGCCCCGGGT	GATTTATTTT	TTTTGTATCT	ACTTCTGACC	CTTTTCAAAT
ATACGGTCAA	CTCATCTTTC	ACTGGAGATG	CGGCCTGCTT	GGTATTGCGA	TGTTGTCAGC
TTGGCAAATT	GTGGCTTTTG	AAAACACAAA	ACGATTCTTT	AGTAGCCATG	CATTTTAAGA
TAACGGAATA	GAAGAAAGAG	GAAATTAAAA	AAAAAATAAA	AACAAACATC	CCGTTCATAA
CCCGTAGAAT	CGCCGCTCTT	CGTGTATCCC	AGTACCACGT	CAAAGGTATT	CATGATCGTT
CAATGTTGAT	ATTGTTCCGC	CAGTATGGCT	CCACCCCAT	CTCCGCGAAT	CTCCTCTTCT
CGAACGCGGT	AGTGGCTGCT	GCCAATTGGT	AATGACCATA	GGGAGACAAA	CAGCATAATA

GCAACAGTGG AAATTAGTGG CGCAATAATT GAGAACACAG TGAGACCATA GCTGGCGGCC
 TGGAAAGCAC TGTTGGAGAC CAACTTGTCC GTTGGGAGGC CAACTTGCAT TGCTGTCAAG
 ACGATGACAA CGTAGCCGAG GACCC

SEQ ID 8

TAA CCG CGG T

SEQ ID 9

CTA GAC CGC GGT TAA T

SEQ ID 10: *T. reesei* *gpd1* promoter

GTCGAC
 ACGATATACA GCGCGGGCTG ATGATAATGA TGATCGAGCA TGACTIONGATG
 CTGTATGTGA CAATATTGAC TGCGAGGAAC CATCAGGTGT GTATGGATGG
 AATCATTCTG TAACCACCAA GGTGCATGCA TCATAAGGAT TCTCCTCAGC
 TCACCAACAA CGAACGATGG CCATGTTAGT GAAGGCACCG TGATGGCAAG
 ATAGAACCAC TATTGCATCT GCGCTTCCCA CGCACAGTAC GTCAAGTAAC
 GTCAAAGCCG CCCTCCCGTA ACCTCGCCCG TTGTTGCTCC CCCCATTGTC
 CTCATCACA TAGTACCTAC CTATGCATTA TGGGCGGCCT CAACCCACCC
 CCCCAGATTG AGAGCTACCT TACATCAATA TGGCCAGCAC CTCTTCGGCG
 ATACATACTC GCCACCCAG CCGGCGCGAT TGTGTGTACT AGGTAGGCTC
 GTACTATACC AGCAGGAGAG GTGCTGCTTG GCAATCGTGC TCAGCTGTTA
 GGTGTGACTT GTATGGTACT TGTAAGGTGG TCATGCAGTT GCTAAGGTAC
 CTAGGGAGGG ATTCAACGAG CCCTGCTTCC AATGTCCATC TGGATAGGAT
 GCGGCTGGC GGGGCCGAAG CTGGGAACCT GCCAACAGTC ATATGTAATA
 GCTCAAGTTG ATGATACCGT TTTGCCAGAT TAGATGCGAG AAGCAGCATG
 AATGTGCTC ATCCGATGCC GCATCACCGT TGTGTCAGAA ACGACCAAGC
 TAAGCAACTA AGGTACCTTA CCGTCCACTA TCTCAGGTAA CCAGGTACTA
 CCAGCTACCC TACCTGCCGT GCCTACCTGC TTTAGTGTTA ATCTTTCCAC
 CTCCCTCCTC AATCTTCTTT TCCCTCCTCT CCTCTTTTTT TTTTCTTCCT
 CCTCTTCTTC TCCATAACCA TTCCTAACAA CATCGACATT CTCTCCTAAT
 CACCAGCCTC GCAAATCCTC AGTTTGTATG TACGTACGTA CTACAATCAT
 CACCACGATC GTCCGCCCCG CGATGCGGCT TCTGTTGCGC TGCCCCCTCCT
 CTCACTCGTG CCCTTGACGA GCTAGCCCCG CCAGGACTCT CCTGCGTCAC
 CAATTTTTTT CCCTATTAC CCCTCCTCCC TCTCTCCCTC TCGTTTCTTC
 CTAACAAACA ACCACCACCA AAATCTCTTT GGAAGCTCAC GACTCACGCA
 GCTCAATTC GCAGATACAA ATCTAGA

SEQ ID 11: *T. reesei* *gpd1* terminator

GGATCC CGAGCATT GTCTATGAAT GCAAACAAAA ATAGTAAATA AATAGTAATT
 CTGGCCATGA CGAATAGAGC CAATCTGCTC CACTTGACTA TCTTGTGACT
 GTATCGTATG TCGAACCCTT GACTGCCCCAT TCAAACAATT GTAAAGGAAT
 ATAGCTACAA GTTATGTCTC ACGTTTGGCT GCGAGCCCGT TTGTACGTTA
 TTTTGAGAAA GCGTTGCCAT CACATGCTCA CAGTCACTTG GCTTACGATC
 ATGTTTGGCA TCTTCGGTAA GAATACACAG AGTAACGATT ATCTCCATCG

CTTCTATGAT TAGGTACTCA GACAACACAT GGGAAACAAG ATAACCATCG
 CATGCAAGGT CGATTCCAAT CATGATCTGG ACTGGGGTAT TCCATCTAAG
 CCATAGTACC CTCGAGAGAA GGAATGGTAG GACCTCTCAG GCGTCCACCA
 TCTGTGCTGC AAATCCAAGA AACCCCCCAA AAGCACCTAC CTATCTACCT
 AGAGTAACTG CACGAGAAAA GAAAAGGAGC AGAAGAAGAA TGATCTCAAG
 AGGCCGTGAA CGCAGAAACA CACTCCTCCC AACTTTTCAA GTTTTGAACA
 AAAAAAGAAA GATGAGGACT AGAAGATGGA GTATTTCCTT CTTAGAGAGC
 TCTCGGTGAG GTGACCTGTC AGGGTTTACC GCAAACCGTC GGTGGTTCTA
 TCCAATTAAT CAAGTCCCGC GCCTCGCCTC TTCTCTCCTG TCCTTTTCATA
 GAATCCCGTC TCCTTGTTGC TTGATCGAAG CGGGGTATC GACGCCACCA
 AAGATCTTGT CTTGGTGACT TATCAATCCT TTGGTGATCA AACAGCCCCC
 GAGTGATCAG ATCCGTAAAA GAAGAAGAAG AGTACGATTT AACCAGACCG
 AGGAACAATA AAGCGAGTAA ATAACATCAA AATAAGAGTC TCGTTGAAAA
 TTAATTGTTT CTCAATCAAT CCCAACCCCC CTAAAAGCCC TTCCCCCAT
 GGTATATCCC GGCAGTAGGA GAGAGATATT TCCACTACCG CTCACCACCA
 AGTGAGGCT TGCCGAGAGA AGAGGATGAA TCAGAAGTGA CAACAACGGG
 TTGAGCACAT GGGATATC GGCGCGCC

SEQ ID12: Sequence of plasmid pAN52-1, 5733 bp

1-2129 *Aspergillus nidulans* *gpdA* promoter
 2130-2304 *A. nidulans* *gpdA*
 2305-3071 *A. nidulans* *trpC* terminator
 3072-5726 pUC18 from *SalI* to *EcoRI*

SQ Sequence 5733 BP; 1435 A; 1454 C; 1378 G; 1463 T; 3 other;

CAATTCCCTT GTATCTCTAC ACACAGGCTC AAATCAATAA GAAGAACGGT TCGTCTTTTT	60
CGTTTATATC TTGCATCGTC CCAAAGCTAT TGGCGGGATA TTCTGTTTGC AGTTGGCTGA	120
CTTGAAGTAA TCTCTGCAGA TCTTTCGACA CTGAAATACG TCGAGCCTGC TCCGCTTGGA	180
AGCGGCGAGG AGCCTCGTCC TGTCACAACCT ACCAACATGG AGTACGATAA GGGCCAGTTC	240
CGCCAGCTCA TTAAGAGCCA GTTCATGGGC GTTGGCATGA TGGCCGTCAT GCATCTGTAC	300
TTCAAGTACA CCAACGCTCT TCTGATCCAG TCGATCATCC GCTGAAGGCG CTTTCGAATC	360
TGTTAAAGAT CCACGTCTTC GGGAAGCCAG CGACTGGTGA CCTCCAGCGT CCCTTTAAGG	420
CTGCCAACAG CTTTCTCAGC CAGGGCCAGC CCAAGACCGA CAAGGCCTCC CTCCAGAACG	480
CCGAGAAGAA CTGGAGGGGT GGTGTCAAGG AGGAGTAAGC TCCTTATTGA AGTCGGAGGA	540
CGGAGCGGTG TCAAGAGGAT ATTCTTCGAC TCTGTATTAT AGATAAGATG ATGAGGAATT	600
GGAGGTAGCA TAGCTTCATT TGGATTGCT TTCCAGGCTG AGACTCTAGC TTGGAGCATA	660
GAGGGTCCTT TGGCTTTCAA TATTCTCAAG TATCTCGAGT TTGAACTTAT TCCCTGTGAA	720
CCTTTTATTC ACCAATGAGC ATTGGAATGA ACATGAATCT GAGGACTGCA ATCGCCATGA	780
GGTTTTCGAA ATACATCCGG ATGTCGAAGG CTTGGGGCAC CTGCGTTGGT TGAATTTAGA	840
ACGTGGCACT ATTGATCATC CGATAGCTCT GCAAAGGGCG TTGCACAATG CAAGTCAAAC	900

GTTGCTAGCA	GTTCCAGGTG	GAATGTTATG	ATGAGCATTG	TATTAAATCA	GGAGATATAG	960
CATGATCTCT	AGTTAGCTCA	CCACAAAAGT	CAGACGGCGT	AACCAAAAGT	CACACAACAC	1020
AAGCTGTAAG	GATTTCCGCA	CGGCTACGGA	AGACGGAGAA	GCCACCTTCA	GTGGACTCGA	1080
GTACCATTTA	ATTCTATTTG	TGTTTGATCG	AGACCTAATA	CAGCCCCCTAC	AACGACCATC	1140
AAAGTCGTAT	AGCTACCAGT	GAGGAAGTGG	ACTCAAATCG	ACTTCAGCAA	CATCTCCTGG	1200
ATAAACTTTA	AGCCTAAACT	ATACAGAATA	AGATAGGTGG	AGAGCTTATA	CCGAGCTCCC	1260
AAATCTGTCC	AGATCATGGT	TGACCGGTGC	CTGGATCTTC	CTATAGAATC	ATCCTTATTC	1320
GTTGACCTAG	CTGATTCTGG	AGTGACCCAG	AGGGTCATGA	CTTGAGCCTA	AAATCCGCCG	1380
CCTCCACCAT	TTGTAGAAAA	ATGTGACGAA	CTCGTGAGCT	CTGTACAGTG	ACCGGTGACT	1440
CTTTCTGGCA	TGCGGAGAGA	CGGACGGACG	CAGAGAGAAG	GGCTGAGTAA	TAAGCCACTG	1500
GCCAGACAGC	TCTGGCGGCT	CTGAGGTGCA	GTGGATGATT	ATTAATCCGG	GACCGGCCGC	1560
CCCTCCGCCC	CGAAGTGGAA	AGGCTGGTGT	GCCCCCTCGT	GACCAAGAAT	CTATTGCATC	1620
ATCGGAGAAT	ATGGAGCTTC	ATCGAATCAC	CGGCAGTAAG	CGAAGGAGAA	TGTGAAGCCA	1680
GGGGTGATA	GCCGTCGGCG	AAATAGCATG	CCATTAACTT	AGGTACAGAA	GTCCAATTGC	1740
TTCCGATCTG	GTAAAAGATT	CACGAGATAG	TACCTTCTCC	GAAGTAGGTA	GAGCGAGTAC	1800
CCGGCGCGTA	AGCTCCCTAA	TTGGCCCATC	CGGCATCTGT	AGGGCGTCCA	AATATCGTGC	1860
CTCTCCTGCT	TTGCCCCGGT	TATGAAACCG	GAAAGGCCGC	TCAGGAGCTG	GCCAGCGGCG	1920
CAGACCGGGA	ACACAAGCTG	GCAGTCGACC	CATCCGGTGC	TCTGCACTCG	ACCTGCTGAG	1980
GTCCCTCAGT	CCCTGGTAGG	CAGCTTTGCC	CCGTCTGTCC	GCCCCGTGTG	TCGGCGGGGT	2040
TGACAAGGTC	GTTGCGTCAG	TCCAACATTT	GTTGCCATAT	TTTCCTGCTC	TCCCCACCAG	2100
CTGCTCTTTT	CTTTTCTCTT	TCTTTTCCCA	TCTTCAGTAT	ATTCATCTTC	CCATCCAAGA	2160
ACCTTTATTT	CCCCTAAGTA	AGTACTTTGC	TACATCCATA	CTCCATCCTT	CCCATCCCTT	2220
ATTCCTTTGA	ACCTTTCAGT	TCGAGCTTTC	CCACTTCATC	GCAGCTTGAC	TAACAGCTAC	2280
CCCGCTTGAG	CAGACATCAC	CATGGATCCA	CTTAACGTTA	CTGAAATCAT	CAAACAGCTT	2340
GACGAATCTG	GATATAAGAT	CGTTGGTGTC	GATGTCAGCT	CCGGAGTTGA	GACAAATGGT	2400
GTTCAGGATC	TCGATAAGAT	ACGTTCATTT	GTCCAAGCAG	CAAAGAGTGC	CTTCTAGTGA	2460
TTTAATAGCT	CCATGTCAAC	AAGAATAAAA	CGCGTTTTCG	GGTTTACCTC	TTCCAGATAC	2520
AGCTCATCTG	CAATGCATTA	ATGCATTGAC	TGCAACCTAG	TAACGCCTTN	CAGGCTCCGG	2580
CGAAGAGAAG	AATAGCTTAG	CAGAGCTATT	TTCATTTTCG	GGAGACGAGA	TCAAGCAGAT	2640
CAACGGTCGT	CAAGAGACCT	ACGAGACTGA	GGAATCCGCT	CTTGGCTCCA	CGGACTATA	2700
TATTTGTCTC	TAATTGTACT	TTGACATGCT	CCTCTTCTTT	ACTCTGATAG	CTTGACTATG	2760
AAAATTCGGT	CACCAGCNCC	TGGGTTTCGA	AAGATAATTG	CATGTTTCTT	CCTTGAACTC	2820
TCAAGCCTAC	AGGACACACA	TTCATCGTAG	GTATAAACCT	CGAAATCANT	TCCTACTAAG	2880
ATGGTATACA	ATAGTAACCA	TGCATGGTTG	CCTAGTGAAT	GCTCCGTAAC	ACCCAATACG	2940
CCGGCCGAAA	CTTTTTTACA	ACTCTCCTAT	GAGTCGTTTA	CCCAGAATGC	ACAGGTACAC	3000
TTGTTTAGAG	GTAATCCTTC	TTTCTAGAAG	TCCTCGTGTA	CTGTGTAAGC	GCCCCACTCA	3060
CATCTCCACT	CGACCTGCAG	GCATGCAAGC	TTGGCACTGG	CCGTCGTTTT	ACAACGTCGT	3120
GACTGGGAAA	ACCCTGGCGT	TACCCAACTT	AATCGCCTTG	CAGCACATCC	CCCTTTCGCC	3180
AGCTGGCGTA	ATAGCGAAGA	GGCCCGCACC	GATCGCCCTT	CCCAACAGTT	GCGCAGCCTG	3240
AATGGCGAAT	GGCGCTGAT	GCGGTATTTT	CTCCTTACGC	ATCTGTGCGG	TATTTACAC	3300
CGCATATGGT	GCACTCTCAG	TACAATCTGC	TCTGATGCCG	CATAGTTAAG	CCAGCCCCGA	3360
CACCCGCCAA	CACCCGCTGA	CGCGCCCTGA	CGGGCTTGTC	TGCTCCCGGC	ATCCGCTTAC	3420
AGACAAGCTG	TGACCGTCTC	CGGGAGCTGC	ATGTGTCAGA	GGTTTTTACC	GTCATCACCG	3480
AAACGCGCGA	GACGAAAGGG	CCTCGTGATA	CGCCTATTTT	TATAGGTTAA	TGTCATGATA	3540
ATAATGGTTT	CTTAGACGTC	AGGTGGCACT	TTTCGGGGAA	ATGTGCGCGG	AACCCCTATT	3600
TGTTTATTTT	TCTAAATACA	TTCAAATATG	TATCCGCTCA	TGAGACAATA	ACCTGATAA	3660

ATGCTTCAAT	AATATTGAAA	AAGGAAGAGT	ATGAGTATTC	AACATTTCCG	TGTCGCCCTT	3720
ATTCCCTTTT	TTGCGGCATT	TTGCCCTTCC	GTTTTTGCTC	ACCCAGAAAC	GCTGGTGAAA	3780
GTAAAAGATG	CTGAAGATCA	GTTGGGTGCA	CGAGTGGGTT	ACATCGAACT	GGATCTCAAC	3840
AGCGGTAAGA	TCCTTGAGAG	TTTTCGCCCC	GAAGAACGTT	TTCCAATGAT	GAGCACTTTT	3900
AAAGTTCTGC	TATGTGGCGC	GGTATTATCC	CGTATTGACG	CCGGGCAAGA	GCAACTCGGT	3960
CGCCGCATAC	ACTATTCTCA	GAATGACTTG	GTTGAGTACT	CACCAGTCAC	AGAAAAGCAT	4020
CTTACGGATG	GCATGACAGT	AAGAGAATTA	TGCAGTGCTG	CCATAACCAT	GAGTGATAAC	4080
ACTGCGGCCA	ACTTACTTCT	GACAACGATC	GGAGGACCGA	AGGAGCTAAC	CGCTTTTTTTG	4140
CACAACATGG	GGGATCATGT	AACTCGCCTT	GATCGTTGGG	AACCGGAGCT	GAATGAAGCC	4200
ATACCAAACG	ACGAGCGTGA	CACCACGATG	CCTGTAGCAA	TGGCAACAAC	GTTGCGCAAA	4260
CTATTAAGTG	GCGAACTACT	TACTCTAGCT	TCCC GGCAAC	AATTAATAGA	CTGGATGGAG	4320
GCGGATAAAG	TTGCAGGACC	ACTTCTGCGC	TCGGCCCTTC	CGGCTGGCTG	GTTTATTGCT	4380
GATAAATCTG	GAGCCGGTGA	GCGTGGGTCT	CGCGGTATCA	TTGCAGCACT	GGGGCCAGAT	4440
GGTAAGCCCT	CCCGTATCGT	AGTTATCTAC	ACGACGGGGA	GTCAGGCAAC	TATGGATGAA	4500
CGAAATAGAC	AGATCGCTGA	GATAGGTGCC	TCACTGATTA	AGCATTGGTA	ACTGTCAGAC	4560
CAAGTTTACT	CATATATACT	TTAGATTGAT	TTAAAACTTC	ATTTTAAATT	TAAAAGGATC	4620
TAGGTGAAGA	TCCTTTTTGA	TAATCTCATG	ACCAAATCC	CTTAACGTGA	GTTTTCGTTC	4680
CACTGAGCGT	CAGACCCCGT	AGAAAAGATC	AAAGGATCTT	CTTGAGATCC	TTTTTTTCTG	4740
CGCGTAATCT	GCTGCTTGCA	AACAAAAAAA	CCACCGCTAC	CAGCGGTGGT	TTGTTTGCCG	4800
GATCAAGAGC	TACCAACTCT	TTTTCCGAAG	GTAAGTGGCT	TCAGCAGAGC	GCAGATACCA	4860
AATACTGTCC	TTCTAGTGTA	GCCGTAGTTA	GGCCACCACT	TCAAGAACTC	TGTAGCACCG	4920
CCTACATACC	TCGCTCTGCT	AATCCTGTTA	CCAGTGGCTG	CTGCCAGTGG	CGATAAGTCG	4980
TGTCTTACCG	GGTTGGACTC	AAGACGATAG	TTACCGGATA	AGGCGCAGCG	GTCGGGCTGA	5040
ACGGGGGGTT	CGTGACACACA	GCCCAGCTTG	GAGCGAACGA	CCTACACCGA	ACTGAGATAC	5100
CTACAGCGTG	AGCTATGAGA	AAGCGCCACG	CTTCCCGAAG	GGAGAAAGGC	GGACAGGTAT	5160
CCGGTAAGCG	GCAGGGTCGG	AACAGGAGAG	CGCACGAGGG	AGCTTCCAGG	GGGAAACGCC	5220
TGGTATCTTT	ATAGTCCTGT	CGGGTTTCGC	CACCTCTGAC	TTGAGCGTCG	ATTTTTGTGA	5280
TGCTCGTCAG	GGGGGCGGAG	CCTATGGAAA	AACGCCAGCA	ACGCGGCCTT	TTTACGGTTC	5340
CTGGCCCTTT	GCTGGCCTTT	TGCTCACATG	TTCTTTCTCTG	CGTTATCCCC	TGATTCTGTG	5400
GATAACCGTA	TTACCGCCTT	TGAGTGAGCT	GATACCGCTC	GCCGCAGCCG	AACGACCGAG	5460
CGCAGCGAGT	CAGTGAGCGA	GGAAGCGGAA	GAGCGCCCAA	TACGCAAACC	GCCTCTCCCC	5520
GCGCGTTGGC	CGATTCATTA	ATGCAGCTGG	CACGACAGGT	TTCCCGACTG	GAAAGCGGGC	5580
AGTGAGCGCA	ACGCAATTAA	TGTGAGTTAG	CTCACTCATT	AGGCACCCCA	GGCTTTACAC	5640
TTTATGCTTC	CGGCTCGTAT	GTTGTGTGGA	ATTGTGAGCG	GATAACAATT	TCACACAGGA	5700
AACAGCTATG	ACCATGATTA	CGAATTGCGG	CCG			5733

SEQ ID 13

GTC AA C CGC GG A CTG CGC ATC ATG AAG TTC TTC GCC ATC

SEQ ID 14

TCT AGC AAG CTT GGC TCT AGT TCT GGA ACC GCA CCA GGC GGC AGC AAC GGC
AAC GGC AAT GTT TGC

SEQ ID 15

TCG TAC AAG CTT TCA AGC ACC GAC GGC GGT

SEQ ID 16

TCT AGC TCT AGA AGC AAC GGC AAC GGC AAT GTT

SEQ ID 17

TGC TAG TCG ACC TGC TAG CAG CAC CGA CGG CGG TCT GSEQ ID 18: *S. cerevisiae* FLO1 coding sequence, 4614 bp

ATGACAATGC CTCATCGCTA TATGTTTTTG GCAGTCTTTA CACTTCTGGC ACTAACTAGT
GTGGCCTCAG GAGCCACAGA GCGGTGCTTA CCAGCAGGCC AGAGGAAAAG TGGGATGAAT
ATAAATTTTT ACCAGTATTC ATTGAAAGAT TCCTCCACAT ATTCGAATGC AGCATATATG
GCTTATGGAT ATGCCTCAA AACCAAATA GGTTCTGTCG GAGGACAAAC TGATATCTCG
ATTGATTATA ATATCCCTG TGTTAGTTCA TCAGGCACAT TTCCTTGTC TCAAGAAGAT
TCCTATGGAA ACTGGGGATG CAAAGGAATG GGTGCTTGTT CTAATAGTCA AGGAATTGCA
TACTGGAGTA CTGATTTATT TGGTTTCTAT ACTACCCCAA CAAACGTAAC CCTAGAAATG
ACAGGTTATT TTTTACCACC ACAGACGGGT TCTTACACAT TCAAGTTTGC TACAGTTGAC
GACTCTGCAA TTCTATCAGT AGGTGGTGCA ACCGCGTTCA ACTGTTGTGC TCAACAGCAA
CCGCCGATCA CATCAACGAA CTTTACCATT GACGGTATCA AGCCATGGGG TGGAAGTTTG
CCACCTAATA TCGAAGGAAC CGTCTATATG TACGCTGGCT ACTATTATCC AATGAAGGTT
GTTTACTCGA ACGCTGTTTC TTGGGGTACA CTTCCAATTA GTGTGACACT TCCAGATGGT
ACCACTGTAA GTGATGACTT CGAAGGGTAC GTCTATTCCT TTGACGATGA CCTAAGTCAA
TCTAACTGTA CTGTCCCTGA CCCTTCAAAT TATGCTGTCA GTACCACTAC AACTACAACG
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ACAACAGCCA CTA CTGCCAT AACTACA ACT GAGCCATGGA ACAGCACTTT TACCTCTACT
TCTACCGAAT TGACCACAGT CACCGGTACC AATGGTTTGC CAACTGATGA GACCATCATT

GTCATCAGAA CACCAACAAC AGCCACTACT GCCATGACTA CAACTCAGCC ATGGAACGAC
 ACTTTTACCT CTACATCCAC TGAAATGACC ACCGTCACCG GTACCAACGG TTTGCCAACT
 GATGAAACCA TCATTGTCAT CAGAACACCA ACAACAGCCA CTACTGCTAT GACTACAAC
 CAGCCATGGA ACGACACTTT TACCTCTACA TCCACTGAAA TGACCACCGT CACCGGTACC
 AACGGTTTGC CAACTGATGA AACCATCATT GTCATCAGAA CACCAACAAC AGCCACTACT
 GCCATGACTA CAACTCAGCC ATGGAACGAC ACTTTTACCT CTACATCCAC TGAAATGACC
 ACCGTCACCG GTACCAATGG TTTGCCAACT GATGAGACCA TCATTGTCAT CAGAACACCA
 ACAACAGCCA CTACTGCCAT GACTACAAC CAGCCATGGA ACGACACTTT TACCTCTACA
 TCCACTGAAA TGACCACCGT CACCGGTACC AACGGTTTGC CAACTGATGA AACCATCATT
 GTCATCAGAA CACCAACAAC AGCCACTACT GCCATAACTA CAACTGAGCC ATGGAACAGC
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 GATGAGACCA TCATTGTCAT CAGAACACCA ACAACAGCCA CTACTGCCAT GACTACAAC
 CAGCCATGGA ACGACACTTT TACCTCTACA TCCACTGAAA TGACCACCGT CACCGGTACC
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 GCCATGACTA CAACTCAGCC ATGGAACGAC ACTTTTACCT CTACATCCAC TGAAATGACC
 ACCGTCACCG GTACCAACGG TTTGCCAACT GATGAGACCA TCATTGTCAT CAGAACACCA
 ACAACAGCCA CTACTGCCAT GACTACAAC CAGCCATGGA ACGACACTTT TACCTCTACA
 TCCACTGAAA TGACCACCGT CACCGGTACC AACGGCGTTC CAACTGACGA AACCGTCATT
 GTCATCAGAA CTCCAAC TAGTGAAGTCTA ATCAGCACCA CCACTGAACC ATGGACTGGT
 ACTTTTACCT CTACATCCAC TGAGATGACC ACCGTCACCG GTACTAACGG TCAACCAACT
 GACGAAACCG TGATTGTTAT CAGAACTCCA ACCAGTGAAG GTTTGGTTAC AACCACCACT
 GAACCATGGA CTGGTACTTT TACTTCTACA TCTACTGAAA TGACCACCAT TACTGGAACC
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 ACCATTACTG GAACCAATGG TCAACCAACT GACGAAACCG TTATTGTTAT CAGAACTCCA
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 TCTACTGAAA TGACCACCGT CACCGGTACC AACGGCGTTC CAACTGACGA AACCGTCATT
 GTCATCAGAA CTCCAACCAG TGAAGGTCTA ATCAGCACCA CCACTGAACC ATGGACTGGC
 ACTTTTCACTT CGACTTCCAC TGAGGTACC ACCATCACTG GAACCAACGG TCAACCAACT
 GACGAAACTG TGATTGTTAT CAGAACTCCA ACCAGTGAAG GTCTAATCAG CACCACCACT
 GAACCATGGA CTGGTACTTT CACTTCTACA TCTGCTGAAA TGACCACCGT CACCGGTACT

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GTTACAACCA CCACTGAACC ATGGACTGGT ACTTTTACTT CGACTTCCAC TGAATGTCT
ACTGTCACTG GAACCAATGG CTTGCCAACT GATGAACTG TCATTGTTGT CAAAACCTCA
ACTACTGCCA TCTCATCCAG TTTGTCATCA TCATCTTCAG GACAAATCAC CAGCTCTATC
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TCCTCAGTAA TTTCTTCCTC AGTCACTTCT TCTCTATTCA CTTCTTCTCC AGTCATTTCT
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TCATCCGTCA TTCCAACCAG TAGTTCACC TCTGGTTCTT CTGAGAGCGA AACGAGTTCA
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ACAAAGCAAA CCAAAGGGAC AACAGAGCAA ACCACAGAAA CAACAAAACA AACACGGTA
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TCTACAAGCA CTGCTACTAT TAACGGCGTT ACTACAGAAT ACACAACATG GTGTCCTATT
TCCACCACAG AATCGAGGCA ACAAACAACG CTAGTTACTG TTACTTCCTG CGAATCTGGT
GTGTGTTCCG AAACGTCTTC ACCTGCCATT GTTTCGACGG CCACGGCTAC TGTGAATGAT
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AAAATGAACA GTGCTACCGG TGAGACAACA ACCAATACTT TAGCTGCTGA AACGACTACC
AATACTGTAG CTGCTGAGAC GATTACCAAT ACTGGAGCTG CTGAGACGAA AACAGTAGTC
ACCTCTTCGC TTTCAAGATC TAATCACGCT GAAACACAGA CGGCTTCGC GACCGATGTG
ATTGGTCACA GCAGTAGTGT TGTTTCTGTA TCCGAACTG GCAACACCAA GAGTCTAACA
AGTCCCGGGT TGAGTACTAT GTCGCAACAG CCTCGTAGCA CACCAGCAAG CAGCATGGTA
GGATATAGTA CAGCTTCTTT AGAAATTTC ACGTATGCTG GCAGTGCCAA CAGCTTACTG
GCCGGTAGTG GTTTAAGTGT CTTCAATTGC TCCTTATTGC TGGCAATTAT TTAA

SEQ ID 19: Sequence of pYES2

Comments for pYES2:

5857 nucleotides

GAL1 promoter: bases 1-452

T7 promoter/priming site: bases 476-495

Multiple cloning site: bases 502-601

CYC1 transcription terminator: bases 609-857

pMB1 (pUC-derived) origin: bases 1039-1712

Ampicillin resistance gene: bases 1857-2717

URA3 gene: bases 2735-3842

2 micron origin: bases 3846-5317

f1 origin: bases 5385-5840

ACGGATTAGAAGCCGCCGAGCGGGTGACAGCCCTCCGAAGGAAGACTCTCCTCCGTGCGTCCTCGTCCTC
ACCGGTCGCGTTCCTGAAACGCAGATGTGCCTCGCGCCGCACTGCTCCGAACAATAAAGATTCTACAATA
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GGTCCCTATTTATTTTTTTATAGTTATGTTAGTATTAAGAACGTTATTTATATTTCAAATTTTCTTTTT
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CGAAGGCTTTAATTTGCGGCCCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCCTATTG
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Claims

1. A process for partitioning of molecules in aqueous two-phase systems (ATPS), comprising the steps of
 - 5 a) constructing a fusion molecule by combining a molecule of interest to a targeting protein having the ability to carry said molecule of interest into one of the phases, and
 - b) subjecting said fusion molecule to an ATPS separation.
2. The process according to claim 1, wherein the targeting protein is a hydrophobic
10 protein.
3. The process according to claim 1, wherein the targeting protein is selected from a group consisting of amphipathic proteins and proteins which form amphipathic aggregates.
15
4. The process according to claim 1, wherein the targeting protein is a hydrophobin-like protein.
5. The process according to claim 4, wherein the hydrophobin-like protein is a class 2
20 hydrophobin.
6. The process according to claim 5, wherein the hydrophobin is a *Trichoderma* hydrophobin.
- 25 7. The process according to claim 6, wherein the *Trichoderma* hydrophobin is HFBII, HFBII or SRHI.
8. A process for partitioning of particles, wherein the particles contain a targeting protein as defined in any one of claims 2 to 7 or a part thereof on their surface, the process
30 comprising the step of
 - subjecting the particles to ATPS separation.
9. The process according to claim 8, wherein the particles are cells.

10. The process according to claim 9, wherein the cells are yeast cells.
11. The process according to claim 8, wherein the particles are spores.
- 5 12. The process according to any one of claims 8 to 11, wherein the targeting protein is fused to a molecule which brings the targeting protein onto the surface of the particle.
13. The process according to any one of claims 1 to 12, wherein the aqueous two-phase system is selected from the group consisting of PEG/salt, PEG/Dextran and PEG/starch
10 systems or derivatives thereof, detergent-based aqueous two-phase systems and thermoseparating polymer systems.
14. The process according to claim 13, wherein the detergent-based ATPS comprises a detergent which is selected from the group consisting of nonionic or zwitterionic
15 detergents.
15. The process according to claim 13, wherein the thermoseparating polymer system comprises a polymer which is selected from the group consisting of polyethylene-polypropylene copolymers.
20
16. The process according to any one of claims 1-15, wherein the molecule of interest or the particle is separated from a suspension containing cells or cell extracts.
17. A fusion molecule, comprising a hydrophobin-like protein as defined in any one of
25 claims 4 to 7 fused to a molecule of interest.
18. A fusion molecule according to claim 17, wherein the molecule of interest is a cell-bound protein or a part thereof.
- 30 19. The fusion molecule according to claim 17, wherein the molecule of interest is an extracellular protein or a part thereof.

20. The fusion molecule according to claim 19, wherein the extracellular protein is an extracellular protein of *Trichoderma*, selected from the group consisting of cellulases, hemicellulases and proteases.

5 21. The fusion molecule according to claim 17, wherein the molecule of interest is an antibody molecule or a part thereof.

22. The process according to claim 1, wherein the targeting protein is fused to the molecule of interest according to any one of the claims 18 to 21.

10

23. A recombinant organism producing a fusion molecule according to any one of claims 17 to 21.

15 24. The recombinant organism according to claim 23, wherein the organism has been genetically modified to be capable of producing a fusion molecule according to any one of claims 17 to 21.

25. A recombinant DNA molecule, comprising a DNA molecule encoding a fusion molecule according to any one of claims 17 to 21.

20

26. A process for producing a targeting protein as defined in any one of claims 4 to 7, or a fusion molecule according to any one of the claims 17 to 21 with recombinant organisms, the process comprising the steps of

25 a) transforming the recombinant organism with DNA molecules enabling expression of such molecules, and

b) recovering such molecules from the culture of the recombinant organism.

27. A process for separating hydrophobin-like molecules in aqueous two-phase systems, the process comprising the steps of

30 a) mixing solutions containing said hydrophobin-like molecule with the phase forming chemicals, and

b) carrying out ATPS separation,

wherein the aqueous two-phase system is as defined in any one of claims 13 to 15.

(57) Abstract

The present invention relates to isolation and purification of proteins in aqueous two-phase systems (ATPS). Specifically the invention provides processes for partitioning of molecules of interest in ATPS by fusing said molecules to targeting proteins which have the ability of carrying said molecule into one of the phases.

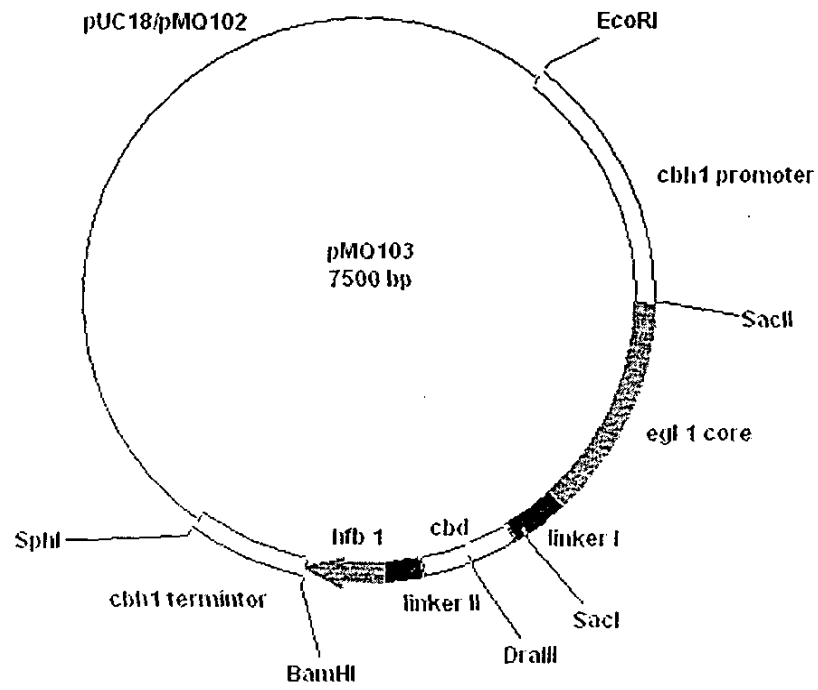


Fig 1.

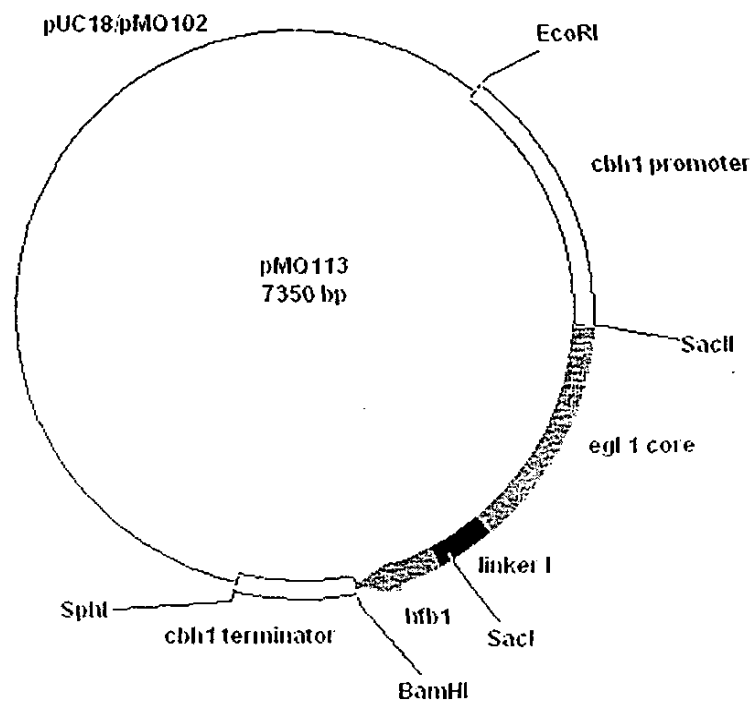


Fig 2.

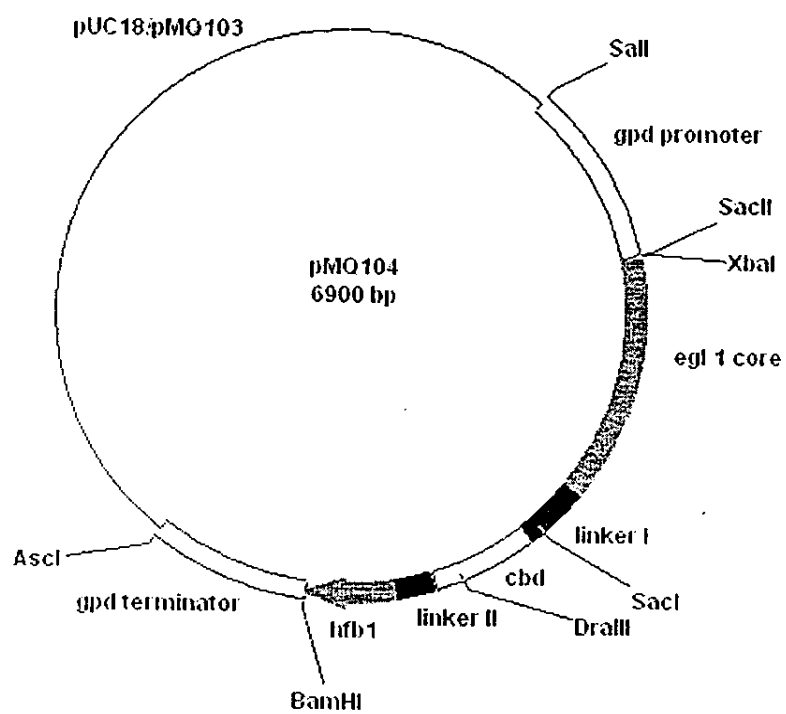


Fig 3.

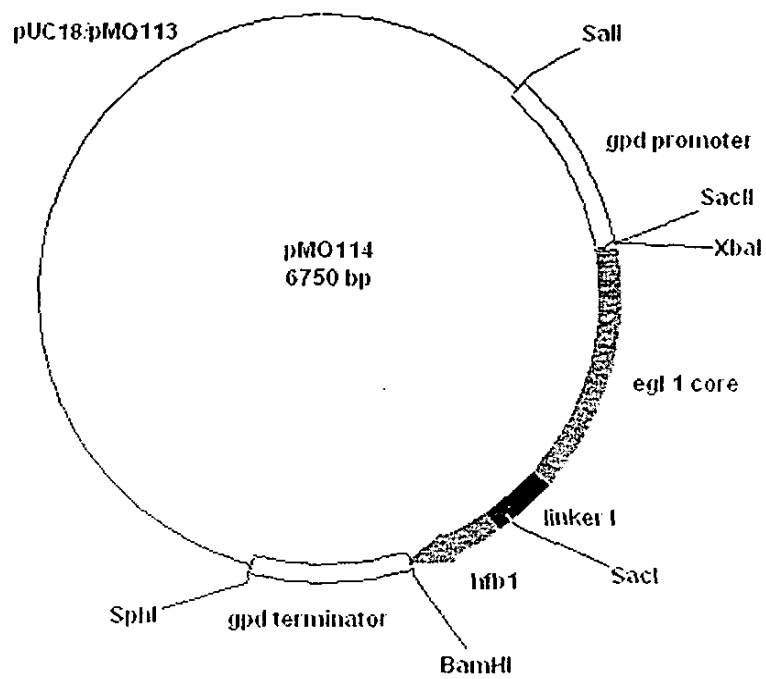


Fig 4.

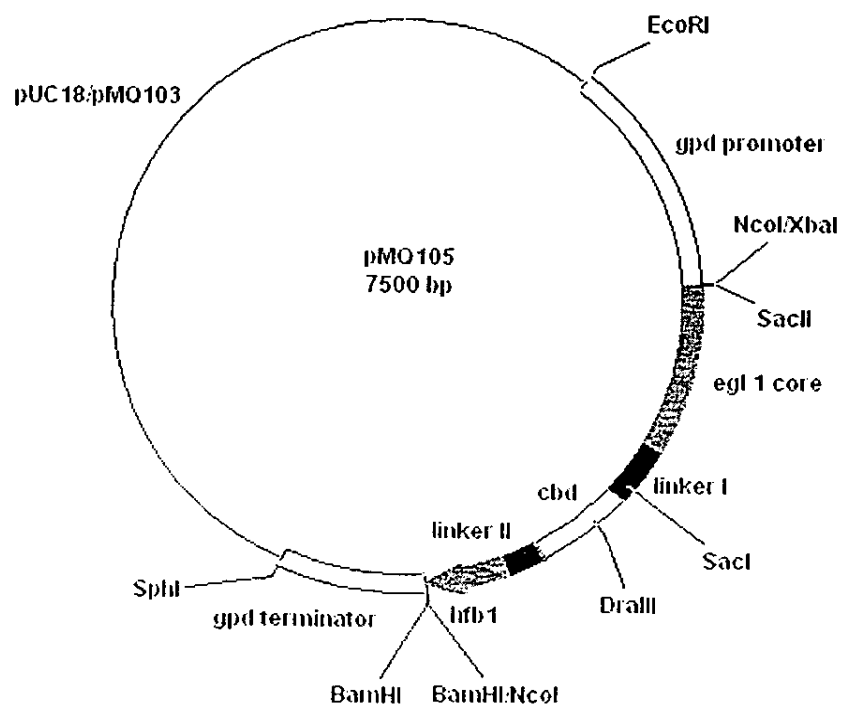


Fig 5.

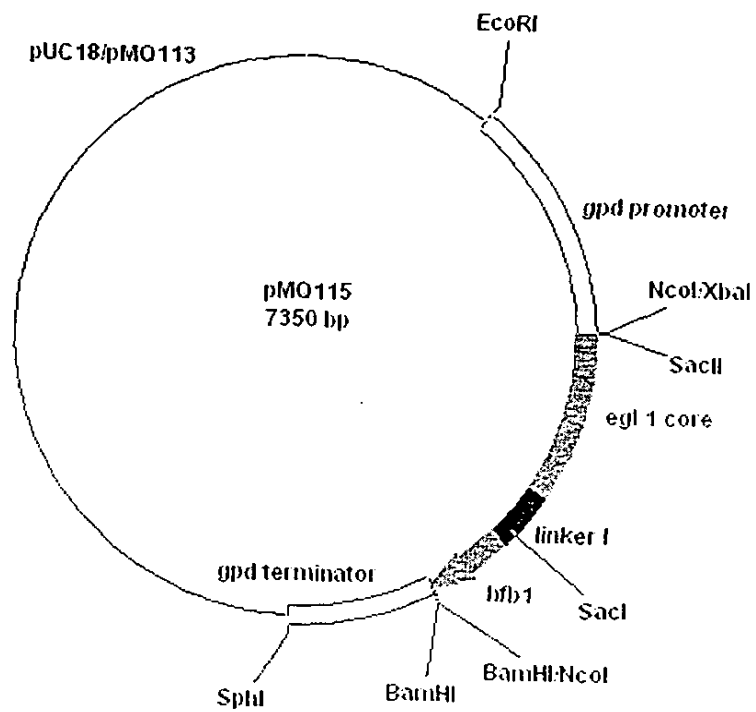


Fig 6.

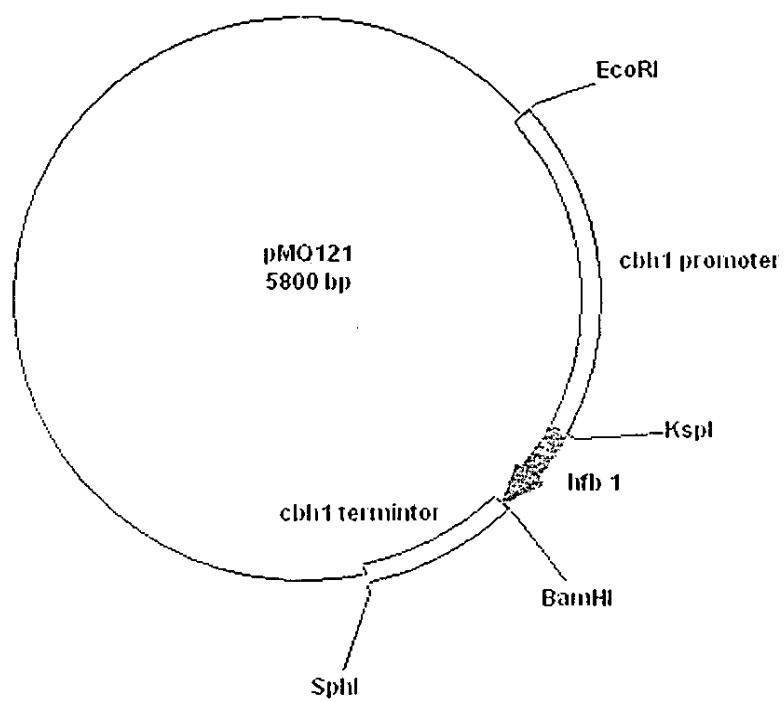


Fig 7.

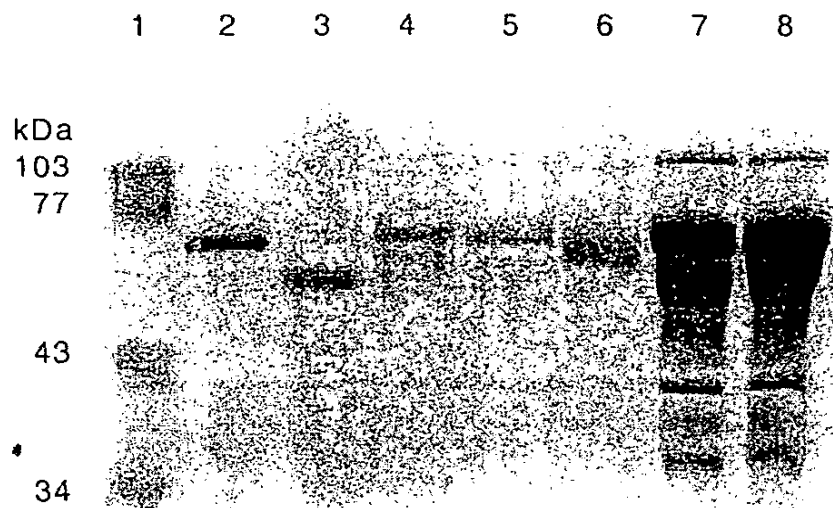


Figure 8. Coomassie stained 10 % SDS-PAGE of the partitioning of EGIcore-HFBI fusion protein in two-phase separation using 5 % of the detergent C12-C18EO5. Lane 1, Molecular weight marker; Lane 2, Purified CBHI (4 μ g); Lane 3, Purified EGI (4 μ g); Lane 4, 1/10 diluted VTT-D-98691 cellulose-based culture filtrate; Lanes 5 and 6, 1/10 diluted bottom phase and detergent phase (top phase), respectively, after separation of VTT-D-98691 culture filtrate with 5% detergent; Lane 7, Non-diluted bottom phase; Lane 8, Non-diluted VTT-D-98691 cellulose culture filtrate.

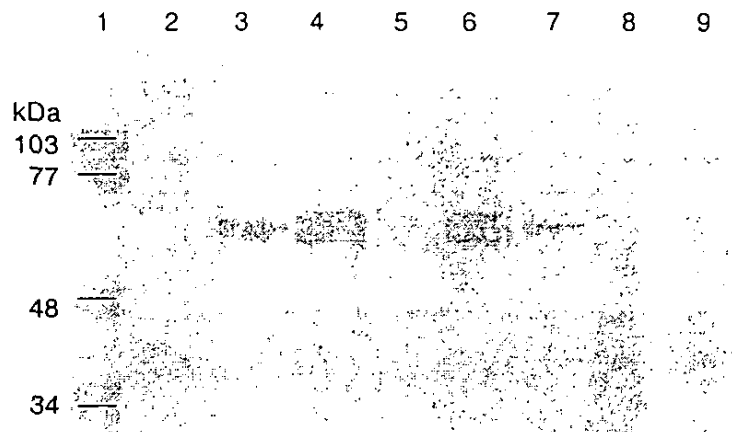


Figure 9. Western analysis of the partitioning of EGIcore-HFBI fusion protein in two-phase separation by using different concentrations of the detergent C12-C18EO5. Fusion proteins were detected with anti-HFBI antibodies. Lane 1, Molecular weight marker; Lane 2, Purified EGI; Lane 3, VTT-D-98691 cellulose culture filtrate; Lanes 4 and 5, Detergent phase (top phase) and bottom phase, respectively, after separation of VTT-D-98691 culture filtrate with 5% detergent; Lane 6, Same as lane 3, except 2 % detergent was used; Lane 7, Same as lane 4, except 2 % detergent was used; Lane 8, Purified EGI; Lane 9:, Purified CBHI.

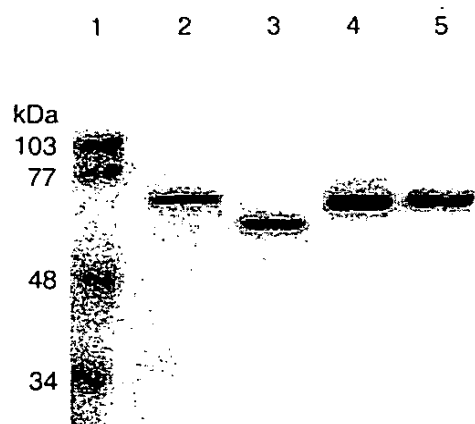


Figure 10. Coomassie stained 10 % SDS-PAGE showing further purification of EGIcore-HFBI fusion protein from the endogenous CBHI when the top phase was re-extracted with 2 % detergent. Lane 1, Molecular weight marker; Lane 2, Purified CBHI (4 μ g); Lane 3, Purified EGI (4 μ g); Lane 4, Detergent phase (top phase) after first extraction; Lane 5, Detergent phase (top phase) after second extraction.

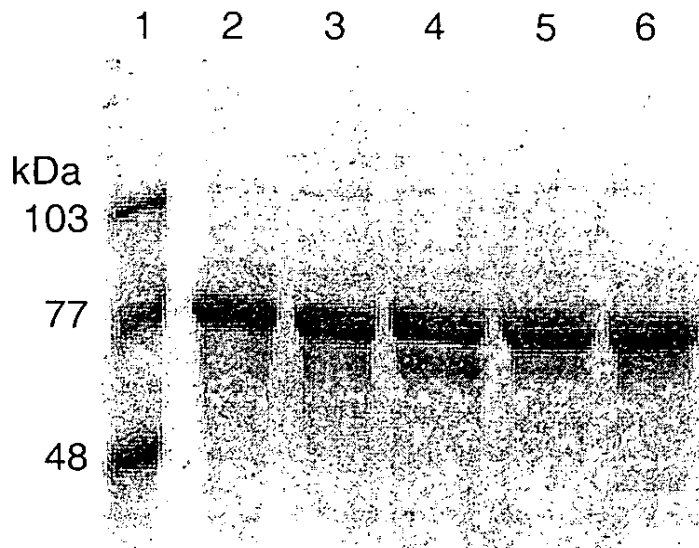


Figure 11. Coomassie stained 10 % SDS-PAGE analysis of the EGI-HFBI protein when treated with thrombin. Lane 1, Molecular weight marker; Lane 2, EGI-HFBI (1 mg/ml) treated 72 h with 3 U of thrombin at 24°C; Lane 3, Same as lane 2, except no thrombin was added; Lane 4, EGI-HFBI (1 mg/ml) treated 48 h with 9 U of thrombin at 36°C; Lane 5, Same as lane 4, except no thrombin was added; Lane 6, Same as lane 5, except no incubation at 36°C.

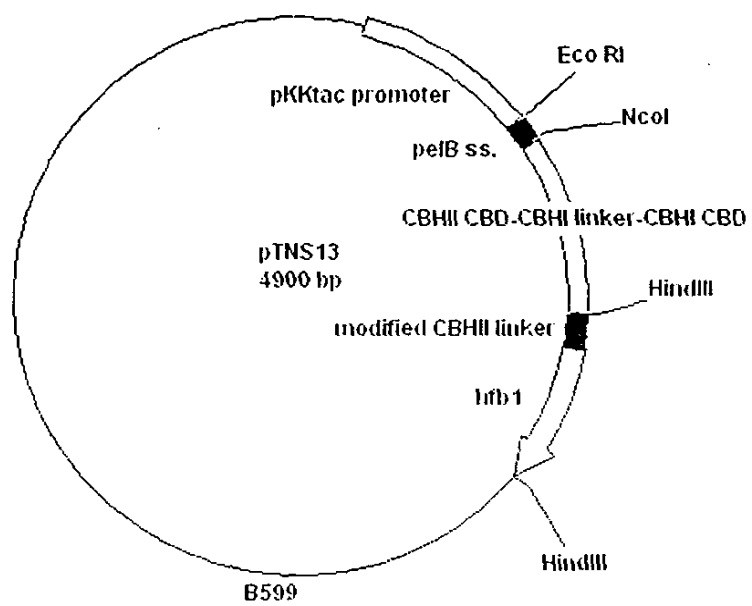
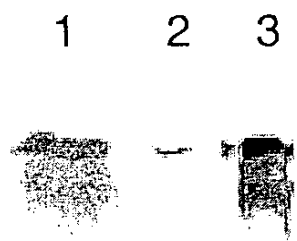


Fig 12.

Figure 13. Western analysis of the partitioning of dCBD-HFB1 fusion protein in two-phase separation using 5 % of the detergent C12-C18EO5. Fusion protein was detected with anti-HFBI antibody. Lane 1, Four times concentrated culture filtrate; Lane 2, Four times concentrated bottom phase; Lane 3, Top phase.



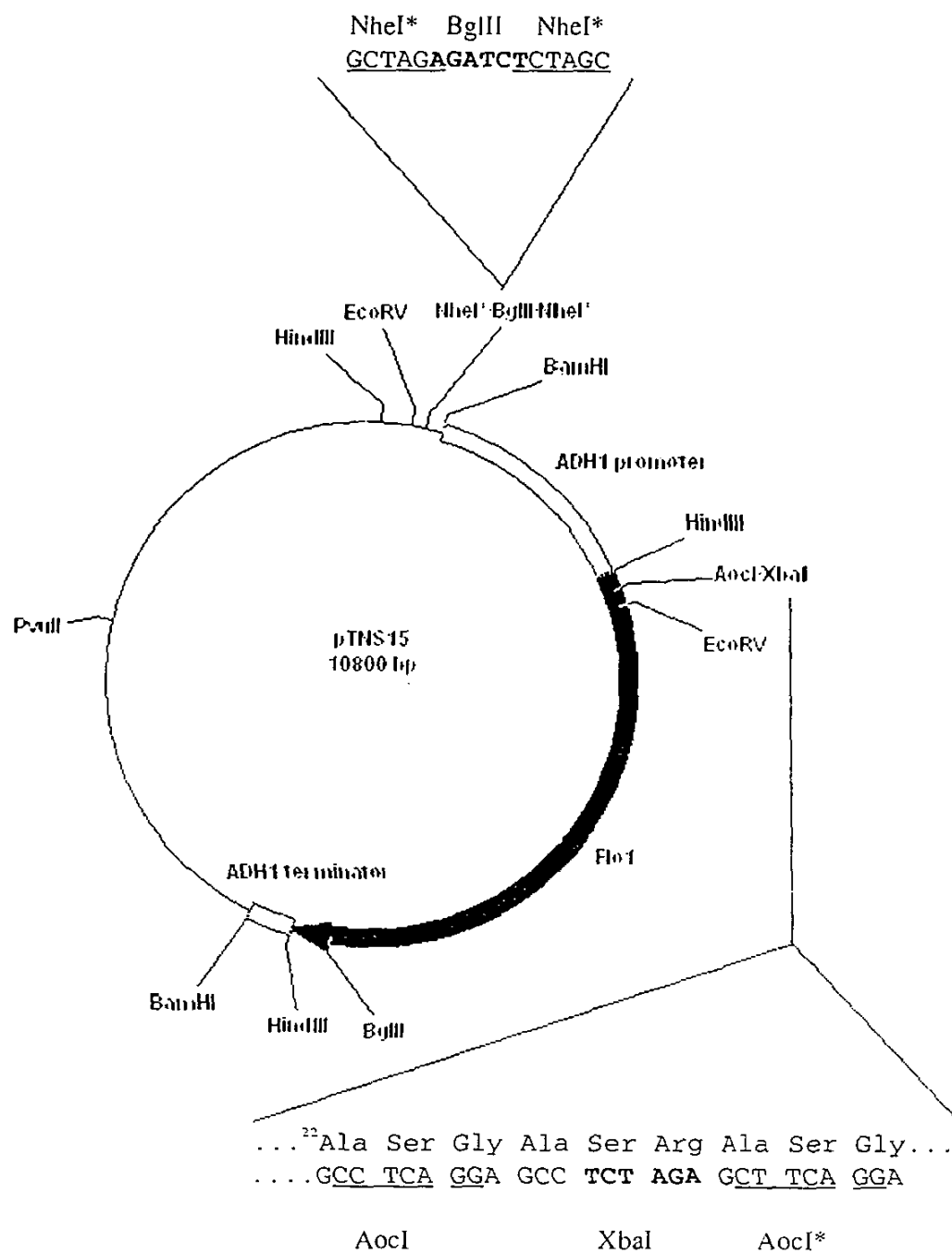


Fig 14.

A non-functional restriction site is indicated with an asterisk.

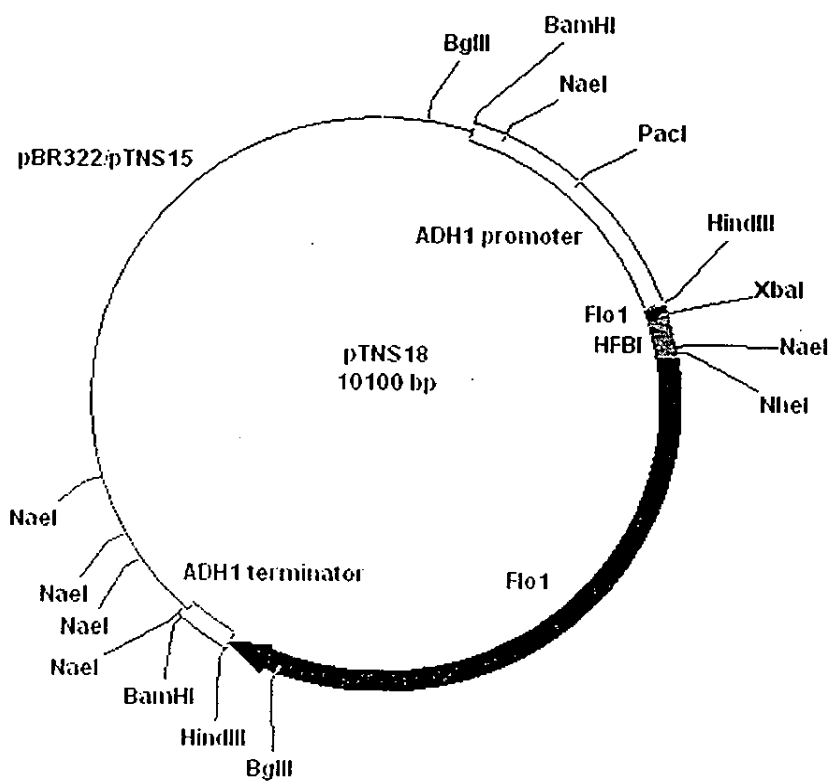


Fig 15.

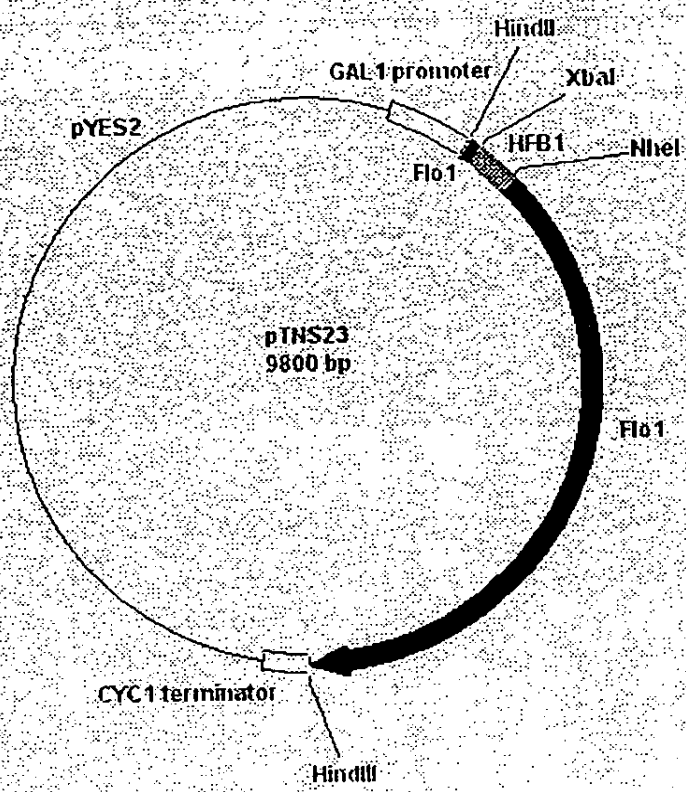


Fig. 16

Figure 17

